Polyurethane Biomaterial-Based Strategies Toward Anti-Infective Gingival Tissue Constructs

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Biomaterials & Biomedical Engineering
University of Toronto

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Abstract

Gingival recession is found in 58% of adults over 30 years old regardless of oral hygiene. Tissue engineering strategies have the potential to overcome the limitations of the current standard of treatment, autologous grafts, among which include tissue shortage and co-morbidity. It is proposed that the gingival connective tissues may be tissue engineered using polyurethane-based scaffold platforms. Polyurethane biomaterials have adaptable chemistry, diverse mechanical properties and suitable biocompatibility and biodegradation characteristics for tissue engineering applications. However, the use of a synthetic material in the infectious oral environment has the potential to lead to biomaterial-associated infections. As such, two different anti-infective scaffold systems will be evaluated in this thesis. In the first system, a degradable polycarbonate urethane (PCNU) was co-electrospun with an antimicrobial oligomer (AO) containing the antibiotic ciprofloxacin (CF) to generate antimicrobial nanofibres. Scaffolds with a higher loading of AO were found to have CF localized on the fibre surfaces which led to a fast release of AO (~100% released within 7 days). Loaded drug was found to slow hydrolysis kinetics of the PCNU scaffolds, and the form of CF loaded was found to significantly affect fibroblast cell attachment, wherein CF incorporated in free form increased cell attachment and spreading on the scaffolds relative to scaffolds with AO. In the second system, a polyurethane-based random pore hydrogel sponge, D-PHI, was used as a scaffold for generating prevascularized gingival constructs via perfusion co-culture of gingival fibroblasts and human vascular endothelial cells (HUVECs) in D-PHI. These constructs are potentially anti-infective in that they will be readily perfused upon implantation so that host defense mechanisms can fight colonization of the
scaffold material by plaque bacteria. In an in vivo subcutaneous model, a portion of engineered microvessels in D-PHI were found to be functional (i.e. perfused with host blood and non-leaky) at 14 days after implantation. The investigation of this system demonstrated the critical importance of perfusion flow toward generating engineered microvessels in vitro. The two strategies find similarities in that they both apply synthetic polyurethane materials with unique surface chemistries, geometries, and mechanical properties that allowed for control over their performance as anti-infective gingival constructs in terms of degradation, drug release, cell attachment, and tissue morphogenesis in in vitro models. The peer-reviewed work from this thesis furthers the field’s understanding of polymer-drug and polymer-cell interactions toward optimizing soft tissue constructs for regenerative medicine.
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List of Abbreviations

AF: Alexa-Fluor.
ANG: angiopoietins.
AO: antimicrobial oligomer.
ASCs: adipose stem cells.
ATR-FTIR: attenuated total reflectance Fourier transform IR spectroscopy.
BCA: bicinchoninic acid.
BD: butane diol.
bFGF: basic fibroblast growth factor.
BPO: benzoyl peroxide.
CF: ciprofloxacin.
CF-DPHI: ciprofloxacin-containing D-PHI.
CFU: colony forming units.
Col I: type I collagen.
DBDL: dibutyltin dilaurate.
DMAC: dimethylacetamide.
DMEM: Dulbecco's modified Eagle's medium.
DMF: dimethylformamide.
D-PHI: degradable, polar, hydrophobic and ionic.
DSC: differential scanning calorimetry.
DVO: divinyl oligomer.
ECM: extracellular matrix.
ECs: endothelial cells.
EGM-2: endothelial cell growth medium-2.
eNO: endothelial nitric oxide.
eNOS: endothelial nitric oxide synthase.
EPCs: endothelial progenitor cells.
EPS: extracellular polymeric substances.
FAK: focal adhesion kinase.
FFT: fast Fourier transform.
FITC: fluorescein isothiocyanate.
FSP: fibroblast surface protein.
GPC: gel permeation chromatography.
GTR: guided tissue regeneration.
H&E: hematoxylin & eosin.
H-bonding: hydrogen bonding.
HDI: hexane diisocyanate.
HEMA: hydroxyethyl methacrylate.
HFIP: hexafluoro-2-propanol.
HGF: human gingival fibroblasts.
HLHCFPEG: hema-lysine-hema with pendant ciprofloxacin-PEG.
HPLC: high performance liquid chromatography.
HUVECs: human vascular endothelial cells.
IgG: immunoglobulin G.
LDI: lysine diisocyanate.
MAA: methacrylic acid.
MIC: minimum inhibitory concentration.
MMA: methyl methacrylate.
MMPs: matrix metalloproteinases.
MSCs: mesenchymal stem cells.
M_w: weight average molecular weight.
M_w,PS: polystyrene equivalent weight average molecular weight.
NV: non-vascularized.
PBS: phosphate buffered saline.
PCL: polycaprolactone.
PCN: polycarbonate.
PCNU: polycarbonate polyurethane.
PDGF: platelet derived growth factor.
PDI: polydispersity index.
PEG: polyethylene glycol.
PGA: polyglycolic acid.
PHD2: prolyl hydroxylase domain 2.
PLGA: polylactic-co-glycolic acid.
PLGF: placentnal growth factor.
PMMA: polymethylmethacrylate.
PTFE: polytetrafluoroethylene.
PV: prevascularized.
ROI: region of interest.
SD: standard deviation.
SDF: stromal cell-derived factor.
SDS: sodium dodecylsulphate.
SE: standard error.
SEM: scanning electron microscopy.
SMA: smooth muscle actin.
TCPS: tissue culture polystyrene.
TEG: triethylene glycol.
TFA: trifluoroacetic acid.
Tg: glass transition temperature.
TGF: transforming growth factor.
Tm: melt transition temperature.
VEGF: vascular endothelial growth factor.
VSMC: vascular smooth muscle cells.
vWF: von Willebrand factor.
XPS: X-ray photoelectron spectroscopy.
ΔH: change in enthalpy.
Chapter 1

1 Introduction

1.1 Motivation

Periodontitis is a chronic inflammatory disease that can lead to tooth loss in adults. Bacterial plaque pathogens initiate a host immune response leading to the destruction of the periodontal tissues that provide structural support for the teeth (Andrian et al. 2007). Periodontitis affects 47% of adults over the age of 30 (Eke et al. 2012). It is especially prevalent in the aging population; 70% of adults over 65 years of age suffer from periodontitis (Eke et al. 2012). Periodontitis is more severe in individuals with Type 1 and Type 2 diabetes, and has been found to have a role in the progression of cardiovascular disease (Southerland et al. 2006). Treatment of periodontitis requires the reduction or elimination of tissue inflammation induced by periodontal bacteria as well as the regeneration of lost tooth-supporting structures. Several different regenerative procedures have been proposed and tested, including guided tissue regeneration, bone and tissue grafting, application of platelet rich plasma, and application of growth factors (Elangovan, Srinivasan, and Ayilavarapu 2009).

Gingival recession is an additional periodontal pathology that can occur due to bacteria-associated periodontal disease or other health factors independent of oral hygiene including trauma, gender, tobacco consumption, or vigorous tooth brushing (Marco C. Bottino et al. 2012). More than half of the population has one or more sites of pronounced gingival recession (Kassab and Cohen 2013). Autologous grafts, taken from the palate or buccal mucosa, are commonly used to repair gingival tissue defects. This surgical procedure has several drawbacks, including increased pain and morbidity at the donor site, potential tissue shortage, and the risk of a loss of original tissue characteristics upon transplantation (Moharamzadeh et al. 2007). Commercial alternatives include epithelial sheets and dermal grafts. Commercially available epithelial sheets are thin, difficult to handle and likely to contract upon implantation. Dermal grafts have not been clinically proven to increase gingival attachment, though they are successfully applied to increase gingival thickness (Moharamzadeh et al. 2012).
Gingival tissue engineering is a promising alternative strategy for regenerating the soft tissue periodontium that has the potential to overcome the limitations of current periodontal regenerative therapies (Benatti et al. 2007). Tissue engineering systems provide the necessary cells as well as the biochemical cues for wound healing and regeneration. Three-dimensional scaffolds guide cell growth and form a template structure. Due to the diffusion limitation of gases, nutrients and waste through tissues, tissue engineered constructs that exceed 200-400 μm in thickness require a blood supply to ensure survival of implanted cells (Rouwkema, Rivron, and van Blitterswijk 2008; Auger, Gibot, and Lacroix 2013). Full-thickness gingival tissue engineered constructs therefore require a means by which the engineered tissues are rapidly vascularized upon implantation. Researchers in the field of tissue engineering are focusing on strategies to prevascularize full-thickness tissue engineered constructs with endothelial cells (ECs) as a means of speeding up angiogenic processes upon implantation.

It is important to note that gingival tissue regeneration strategies involving synthetic scaffold materials must incorporate a strategy for combatting the infectious oral environment, which has been shown to inhibit regeneration in other periodontal tissue regeneration strategies involving synthetics (Bottino et al. 2012). The phrase “the race to the surface” was coined to summarize the phenomenon by which bacteria may colonize a biomaterial implant and lead to implant-associated infection if the host’s own cells do not first attach and integrate into the implant (Gristina 1987). Tissue integration in general and vascularization of the implant in particular are required for the natural host defenses to prevent the infection of implanted materials. Inflammatory cytokines and chemokines in the tissue local to the implant direct inflammatory cells to migrate towards the infection site via the vasculature and participate in infection clearance and wound healing. For gingival constructs less than 200 μm in thickness, such as those fabricated using fibrous mats and single-cell layers, prevascularization with ECs is not required. However, an alternative approach to ensuring the host’s own cells and tissue win the “race to the surface” of the scaffold implant is required. In these cases, an anti-infective scaffold material may be used.

In the current thesis, two different gingival tissue engineering scaffold systems are evaluated for use in gingival tissue regeneration. In the first system, a degradable polycarbonate polyurethane (PCNU) (Tang, Labow, and Santerre 2001a, 2001b) was co-electrospun with an
antimicrobial oligomer (AO) containing the antibiotic ciprofloxacin (CF) to generate antimicrobial PCNU nanofibres. The AO, which was provided by Interface Biologics Inc. (Toronto, Canada), was incorporated at 7 and 15% w/w equivalent CF, and compared to fibres with 15% w/w free CF. In the second system, a prevascularized gingival construct was fabricated via co-culture of human gingival fibroblasts (HGF) and human vascular endothelial cells (HUVECs) on drug-free degradable, polar, hydrophobic and ionic (D-PHI) polyurethane scaffolds fabricated via free radical polymerization as per methods reported elsewhere (Cheung, Rose, and Santerre 2013). Co-cultures on D-PHI scaffolds were cultured in a perfusion bioreactor to promote HUVEC organization into microvessel structures, as a prevascularized construct may allow for accelerated blood perfusion upon implantation, enabling the natural host defense system to take effect.

1.2 Background

1.2.1 Antimicrobial Scaffolds for Anti-infective Tissue Engineering

Independently of tissue engineering, polymer-based antibacterial (or antimicrobial) delivery systems have been explored as a means of delivering drug locally and in a controlled system. Although a high concentration of antibiotic at the time of implantation is desired so as to prevent initial bacterial contamination of the scaffold, the concentration must not be so high as to cause a cytotoxic effect for the local host cell population. Furthermore, release must continue beyond this point at a concentration that is above the minimum inhibitory concentration (MIC) of potential pathogens. Prolonged release of antibiotic below the MIC may favour the growth of antibiotic-resistant strains of bacteria and make any infection more difficult to treat.

For soft tissue engineering applications, electrospinning blends of polymers and antibiotics is a particularly attractive approach for fabricating anti-infective scaffolds for tissue regeneration, as antibiotics and polymers can be blended and electrospun into highly porous mats in a straightforward manner. However, poor antibiotic-polymer physical interactions often leads to microstructural configurations that result in a burst release of antibiotics (Rambhia and Ma 2015). This excludes their usefulness for longer-term applications. The rate of drug release has been shown to vary with different antibiotics and the form in which they are loaded (e.g. salt form or acid/base form) (Gilchrist et al. 2013b; Toncheva et al. 2012), the drug loading (Cui et
al. 2006; Gilchrist et al. 2013b; Natu, de Sousa, and Gil 2010; Peng et al. 2008; Weldon et al. 2012), as well as with the addition of defined additives such as polyethylene glycol (PEG) (Toncheva et al. 2012; K. Kim et al. 2004). Coaxial electrospinning, emulsion electrospinning and drug-loaded nanoparticle additives have all been used in an effort to slow down the release of drug molecules from electrospun scaffolds, with some success (Ignatova et al. 2013).

Partly to address the issue of non-ideal antibiotic-polymer physical interactions, a novel oligomeric monomer containing the fluoroquinolone antibiotic ciprofloxacin was developed recently for application in dental resins for the prevention of secondary caries (Delaviz, Liu, et al. 2018; Delaviz, Nascimento, et al. 2018). The ciprofloxacin that is released via hydrolysis is covalently linked to the polymer backbone as a pendant CF/PEG molecule such that the bioactive biodegradation by-products are limited to CF or CF/PEG.

1.2.2 Prevascularization of Tissue Engineering Constructs

Strategies for engineering microvessels in a biomaterial scaffold generally involve engineering a sophisticated scaffold material that is pro-angiogenic. This may be accomplished via the incorporation of geometric cues (Mirabella et al. 2017), mechanical cues (Y. C. Chen et al. 2012), or pro-angiogenic biomolecules such as growth factors (Amaral et al. 2018) and extracellular proteins (Landau et al. 2017). It is also common to employ a co-culture technique, as ECs are widely reported to fail to organize or survive in a construct long-term without supporting cells such as fibroblasts, mesenchymal stem cells (MSCs) or other tissue-specific cells (Battiston et al. 2014). Scaffolds with biomolecules are expensive and difficult to standardize or customize to meet different clinical needs, while micropatterned scaffolds have shown mixed success in the literature and do not necessarily replicate the arbitrary and multidimensional network of native microvasculature in an adequate manner (Morgan et al. 2019). Synthetic scaffolds are more adaptable and practical for translation to the clinic; however, when synthetic scaffold materials (e.g. polylactic-co-glycolic acid, PLGA) are used, it is common to pre-treat the scaffold with fibronectin (Santos et al. 2009; Bertlein et al. 2018), seed the cells within a fibrin matrix (Mirabella et al. 2017), or even functionalize the scaffold with relevant biomolecules (Landau et al. 2017).
Culturing the cell-seeded scaffolds under dynamic culture conditions, i.e. within a perfusion bioreactor, can activate shear-induced biochemical signalling pathways to promote EC invasion and organization (Kaunas, Kang, and Bayless 2011). Perfusion of growth medium also improves diffusion of gases (oxygen) and small molecules (glucose), and significantly affects protein and macromolecule gradients within tissue matrices via convection, and hence can guide angiogenesis processes (Shirure et al. 2017; Griffith and Swartz 2006). Finally, hypoxic culture conditions may also be employed as a means of encouraging EC self-assembly via hypoxia-induced signalling pathways, though only a small number of studies have thoroughly investigated how oxygen gradients within the tissue matrix or scaffold affect angiogenesis (Phillips, Birnby, and Narendran 1995; Ottino et al. 2004).

1.3 Central Objective and Hypothesis

The central objective of this thesis was to evaluate two defined anti-infective gingival tissue engineering strategies: (1) Blend electrospinning of PCNU and the CF-containing AO in nanofibre scaffolds evaluated in terms of sustainment of drug release, antibacterial efficacy, and in vitro cell compatibility; and (2) Prevascularization of D-PHI sponge scaffolds via perfused co-cultures of HGF and HUVECs, evaluated in terms of the extent of blood perfusion and tissue integration within the construct once implanted in vivo.

It is hypothesized that (1) incorporating antibiotic via blend electrospinning of AO with PCNU will promote a uniform distribution of drug throughout the scaffolds such that as the scaffold matrix degrades by hydrolysis, there will be a sustained release of antibiotic with antibacterial activity against characteristic microorganism *Porphyromonas gingivalis* without compromising fibroblast cell compatibility; and (2) in vitro perfused co-culture of HGF and HUVECs on D-PHI will result in tissue engineered constructs that mimic crucial aspects of the natural biology of the human gingival connective tissues (synthesis of type I collagen and formation of EC lumen structures) so as to promote tissue integration and blood perfusion of the tissue constructs upon implantation in vivo.
## 1.4 Objectives

### 1.4.1 Objective 1

Incorporate the AO into electrospun PCNU nanofibre scaffolds and evaluate suitability of the scaffolds for use as an anti-infective gingival tissue engineering scaffold platform in terms of drug release, scaffold degradation rate, antimicrobial efficacy of the scaffolds, and HGF compatibility. *(Addresses Hypothesis 1.)*

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<th><strong>Approach</strong></th>
<th><strong>Rationale</strong></th>
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<td>Blend electrospinning techniques were used to fabricate PCNU and AO nanofibres at varying concentrations of AO (7 and 15% w/w equivalent CF).</td>
<td>The AO was used as a means to slow release of bioactive CF from the scaffolds by increasing chemical compatibility between the PCNU matrix and the drug, and because the AO must be hydrolyzed before it has bioactivity (Marsac, Shamblin, and Taylor 2006; Krause 1978). The AO was not covalently bonded to the bulk polymer but rather blended through physical interactions as previous investigations revealed that covalently bonding CF into the polymer resulted in a broad range of potentially bioactive degradation by-products, which is not ideal for practical purposes (Woo, Mittelman, and Santerre 2000). Blend electrospinning of PCNU was used to generate nanofibres as this is a straightforward method of incorporating adjuncts into nanofibres that avoids the complexity of co-axial electrospinning, or additional fabrication steps and additives as required with emulsion electrospinning (Murugan and Ramakrishna 2007). Two different formulations were characterized as drug release rate has been shown to be dependent on drug-loading (Cui et al. 2006; Gilchrist et al. 2013b; Natu, de Sousa, and Gil 2010; Peng et al. 2008; Weldon et al. 2012).</td>
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Physical properties of the scaffolds were characterized (i.e. fibre morphology, polymer microstructure, scaffold surface chemistry, and drug distribution).

The scaffold architecture is a great determinant of the scaffold’s performance *in vivo*. Porosity and pore interconnectivity promote the diffusion of nutrients, waste, and cells throughout the scaffold (Ma 2004; Yang et al. 2001). Fibre diameter affects cell attachment (Pham et al. 2006), while fibre alignment promotes cell alignment and may also yield a more desirable fibroblast phenotype by promoting the production of extracellular matrix (ECM) molecules (Johnson et al. 2006; C. H. Lee et al. 2005). For the given application, scaffolds with aligned fibres and an average fibre diameter of 200-300 nm were desired, and these features were maintained between scaffolds with varying concentrations of AO or CF by controlling the electrospinning parameters. Similar fibre morphology with electrospun PCNU has previously been shown to form a suitable substrate for annulus fibrosis cell adhesion, phenotype maintenance and ECM accumulation (Yeganegi, Kandel, and Santerre 2010; Iu, Santerre, and Kandel 2014).

The polarity of a scaffold is another important property that is known to influence cell attachment (Yamamoto et al. 2000; Satriano et al. 2003; Hallab et al. 2001). As such, the polar character of the scaffolds was measured by determining the water contact angle.

The influence of the added AO and CF on hydrogen-bonding within the PCNU was determined both by chemical characterization (attenuated total reflectance Fourier transform IR spectroscopy (ATR-FTIR)) and physical characterization (differential scanning calorimetry (DSC)). DSC provided information on the polymer microstructure,
which is largely determined by intermolecular hydrogen bonding between the soft- and hard-segments of the PCNU. Hydrogen bonding in the polymer is an important factor affecting PCNU degradation rate (Tang, Labow, and Santerre 2001a), and the degradation rate of the PCNU was expected to affect the suitability of the scaffold for the proposed application in terms of both tissue integration and drug release.

The distribution of the AO and CF within the scaffold fibres was also visualized using confocal laser scanning microscopy, wherein autofluorescent CF provided a signalling molecule in contrast to the PCNU. Since polar drugs have been shown to segregate to the surface of fibres during blend electrospinning (K. Kim et al. 2004; Natu, de Sousa, and Gil 2010; He, Huang, and Han 2009), surface-resolved X-ray photoelectron spectroscopy (XPS) was used to measure the fluorine concentration in the scaffolds (fluorine is present in CF and not the PCNU) at various depths into the fibres.

**Scaffold drug release and degradation rate were investigated.**

Scaffold drug release and degradation rate were investigated in a simple chemical aqueous *in vitro* model in order to assess the suitability of the scaffolds as tissue engineering scaffolds with prolonged drug release. Scaffold sections were incubated in a phosphate-buffered solution at 37°C mimicking physiological conditions for 28 days. These conditions have previously been shown to result in measurable PCNU scaffold degradation (Yeganegi, Kandel, and Santerre 2010), and also represent a physiologically suitable timeframe for drug release (Sill and von Recum 2008). Therefore, the scaffold degradation rate needed to be
investigated for any changes that may affect its suitability for a drug release and tissue engineering scaffold. The concentrations of AO and CF in the incubation solutions were determined via high performance liquid chromatography (HPLC). The extent of scaffold degradation was determined by measuring the change in molecular weight of the PCNU via gel permeation chromatography (GPC).

The antimicrobial efficacy of the scaffold release products was measured, and the cell compatibility was assessed. To ensure that the processing of the AO and CF into the scaffolds did not affect the antimicrobial efficacy of the drug, the MIC of release products against cultures of characteristic organism *P. gingivalis* was measured and compared to off-the-shelf CF. The cytotoxicity of the scaffolds and the degradation by-products were determined by culturing with HGF, the cells that are responsible for the maintenance and remodelling of the connective tissue (Taba et al. 2005; Giannopoulou and Cimasoni 1996), and are used to make oral mucosa constructs (Moharamzadeh et al. 2012). A live/dead assay was used to determine the viability of attached cells, scanning electron microscopy (SEM) was used to determine the cell morphology of attached cells, and a WST-1 assay was used to determine the viability of cells exposed to release products.

**Related Papers:**

• The study described in this paper was conceived, executed, and reported by me. Co-author Ian Parrag contributed knowledge transfer for purification and characterization of the AO. Meilin Wang contributed training on electrospinning equipment and PCNU synthesis. JP Santerre was the senior author.


• The study described in this paper was conceived, executed, and reported by me. Co-author Andrew Wong completed some cytotoxicity experiments with scaffold degradation components and HGF. Daniel Levitt completed some scaffold antimicrobial efficacy experiments with *P. gingivalis*. Additional authors had roles as described for related paper (1).

1.4.2 Objective 2

Investigate the use of *in vitro* perfusion flow culture using HGF and HUVEC co-cultures within porous D-PHI scaffolds as a means of engineering gingival constructs with a pre-established microvessel network that anastomoses with the host vasculature once implanted *in vivo* and promotes tissue integration. (*Addresses Hypothesis 2.*)

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<td>HGF and HUVECs within D-PHI scaffolds were co-cultured in custom-built perfusion bioreactors under 3 different flow rates to determine an optimal flow rate in terms of promoting tissue deposition and microvessel organization within the constructs.</td>
<td>D-PHI has previously been used in tissue engineering applications (Sharifpoor, Labow, and Santerre 2009; X. Zhang et al. 2017). It has a unique heterogenous surface chemistry that was shown to encourage a pro-wound healing phenotype associated with macrophages (K. G. Battiston et al. 2015). HUVECs were used as a source of ECs in combination with HGF, as ECs alone often fail to organize or survive in a construct long-term without supporting cells (K. G. Battiston et al., 2014). HGF are also highly synthetic</td>
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and produce ECM tissue, vital for tissue morphogenesis and for enhancing the integration of the construct upon implantation.

Perfusion bioreactors have been shown to enhance cell proliferation, metabolic activity, and type I collagen production of HGF on D-PHI scaffolds (Cheung, Rose, and Santerre 2013). Mechanical stress can activate or inhibit specific molecular pathways (e.g. Smad 2/3 (Hinz et al. 2007) and focal adhesion kinase (FAK)/mTOR (Dalla Costa et al. 2010)) and cause different cell responses (e.g. myofibroblast differentiation (Hinz et al. 2007) and EC invasion and organization (Kaunas, Kang, and Bayless 2011)). Increased production of pro-angiogenic factors transforming growth factor (TGF)-β1 and vascular endothelial growth factor (VEGF) by HGF cells is expected to promote HUVEC lumen formation in the scaffolds (Sorrell, Baber, and Caplan 2007).

Three different magnitudes of flow rate were investigated such that three different shear stress ranges were approximated to be experienced by cells, including physiological interstitial flow shear, an intermediate shear, and shears experienced by ECs in capillaries (sprouting shears) (Chary and Jain 1989; Kaunas, Kang, and Bayless 2011) (see Chapter 5.7 Supplementary Data). Constructs generated at each flow rate were evaluated in terms of cell proliferation, production of type I collagen, and organization of the HUVECs into capillary-like structures.

| Constructs generated at an optimal flow rate magnitude | A typical immunocompromised athymic mouse xenograft model was used. A subcutaneous wound was used in place |
were implanted in a subcutaneous xenograft mouse model. The constructs were surgically exposed via a dorsal window for intravital imaging. The constructs were surgically exposed via a dorsal window for intravital imaging. Intravital imaging was used to show the perfusion of host blood through the engineered microvessels in order to validate that engineered vessels anastomosed with the host vessel system and remained patent over the study time. The construct was immobilized in a stainless-steel dorsal window for the purpose of imaging.

Tissue integration and neovascularization within the constructs was assessed using end-point histological techniques and compared to non-prevascularized controls. Control constructs that were not prevascularized were implanted on the opposite side of the mouse’s back to the prevascularized constructs. The controls were D-PHI scaffolds with HGF and HUVECs at similar ratios and cell numbers but no defined lumen structures. While the majority of the literature emphasizes the importance of prevascularization for tissue engineering strategies, some studies have shown that EC organization in vitro prior to implantation does not necessarily improve the vascularization of the implant (Lin et al. 2017). End-point excision and tissue fixation followed by tissue staining using histological staining was used to assess the effectiveness of the prevascularization strategy in terms of tissue deposition and vessel lumen density within the construct.

**Related Paper:**

**Wright, MEE, Maeda, A, Yeh, SA, DaCosta, RS, Lin, CP & Santerre, JP.** Harnessing prefusion flow in in vitro co-cultures for engineering functional microvessels in synthetic polyurethane
random-pore scaffolds. Under revisions for resubmission to *Biomaterials* (expected submission date September 2019).

- The study described in this paper was conceived, executed, and reported by me. Co-author Azusa Maeda helped with the dorsal window study design. Allison Yeh contributed expertise in live tissue staining. Senior author RS DaCosta contributed research facilities in Toronto for the mouse studies, while senior author CP Lin contributed imaging and mouse facilities in Boston. JP Santerre was the lead principal investigator and holds the grants that funded the project.

### 1.5 Additional Contributions

In addition to the above contributions to the literature, I also collaborated on a skin tissue engineering study with a post-doctoral fellow at Sunnybrook Hospital’s Laboratory of Burn Research and Skin Regeneration. This project allowed me to apply my expertise in electrospinning of PCNU to a new tissue engineering strategy toward skin substitutes to treat burn wounds. A paper has been submitted (Sheikholeslam, M., **Wright, M. E. E.**, Cheng, N., Oh, H. H., Wang, Y., Datu, A., Santerre, J. P., Amini-Nik, S. & Jeschke, M. G. Electrospun co-Polyurethane-Gelatin Composite: A New Tissue Engineered Scaffold for Application in the Skin Regeneration and Repair of Complex Wounds. Submitted to *ACS Biomaterials Science & Engineering* June 2019). I also contributed to a related review paper on biomaterials for skin substitutes (Sheikholeslam, M., **Wright, M. E. E.**, Jeschke, M. G. & Amini-Nik, S. Biomaterials for Skin Substitutes. *Adv. Healthc. Mater.* 7, 1–20 (2018)).

In a collaborative project with Dr. Yasaman Delaviz, a colleague in the Santerre Lab, I fabricated D-PHI scaffolds modified with an antimicrobial monomer previously synthesized and characterized by Delaviz et al. (Delaviz, Nascimento, et al. 2018). My specific contributions included fabricating the scaffolds, visualising the distribution of antimicrobial in the scaffolds using confocal microscopy, characterizing the drug release rate in an *in vitro* model, and assessing the compatibility of the scaffolds with HGF. This work was presented in 2017 at the Society for Biomaterials in Minneapolis, United States, and is summarized in Appendix A.
Chapter 2

2 Literature Review

2.1 Gingival Tissue Engineering

2.1.1 Gingival tissue anatomy, physiology and disease

Gingival tissue, otherwise known as the gingiva or gum tissue, is a part of the oral mucosa that surrounds the teeth and covers the mandibular and maxillary bones. The gingiva is one of the four tissues that comprise the periodontium, the group of tissues that anchors the teeth in the mandible and maxilla. The periodontal tissues consist of epithelial tissue as well as soft and mineralized connective tissue. These include: (1) the alveolar bone, the bone process that contains the tooth socket; (2) the periodontal ligament, the organized connective tissue fibres that allow for structural attachment of the tooth to the alveolar bone; (3) the cementum, the mineralized tissue that attaches the tooth to the alveolar bone via the periodontal ligament; and (4) the gingiva, the mucosal tissue that provides a seal around the tooth and acts as a barrier to bacterial or physical assaults to the deeper and more structural tissues of the periodontium (Bottino et al. 2012) (Figure 2-1A). In adults, the gingival tissue ranges from 1-10 mm in thickness and 5-7 mm in height (Goaslind et al. 1977).

Figure 2-1. (A) The tissues comprising the periodontium – the group of tissue that provides anchorage and structural support for the teeth. Adapted from (Edwards and Kanjirath 2010). (B) The periodontal tissue blood supply. Adapted from (Schroeder and Listgarten 1997).
The gingiva consists of epithelial tissue supported by a fibrous connective tissue layer, termed the lamina propria (Moharamzadeh et al. 2007). Similar tissue is found on the tongue and hard palate. The average healthy gingiva consists of about 30% epithelium and 70% lamina propria connective tissue (Schroeder and Listgarten 1997). The epithelial layer is stratified squamous epithelium, which may be keratinized or nonkeratinized, depending on its location (Moharamzadeh et al. 2007; Locke et al. 2008). The epithelium is attached to the lamina propria through the basement membrane, which contains type IV collagen, and adhesive molecules laminin, and fibronectin. The lamina propria itself contains a network of type I collagen, type III collagen, and elastin, as well as vasculature components, lymphatic vessels, and salivary ducts and glands (Moharamzadeh et al. 2007). About 60% of the connective tissue volume is taken up by the collagen network, and of this fraction, 90% of the volume is type I collagen fibre bundles (Schroeder and Listgarten 1997).

The major cellular component of the lamina propria are the gingival fibroblasts, which exist in the connective tissue at a density of about 200 million cells/cm³ (Schroeder and Listgarten 1997). Fibroblasts are responsible for the maintenance and remodelling of connective tissue ECM. Fibroblasts synthesize ECM substances and produce enzymes capable of ECM remodelling. During wound repair, they synthesize an abundance of types I, III, and IV collagen, as well as fibronectin and heparin sulphate proteoglycans (Hughes 2008; Hillmann, Gebert, and Geurtsen 1999). Fibroblasts are also responsible for the synthesis of important enzymes such as matrix metalloproteinases (MMPs) and serine proteinases (Schoen and Mitchell 2013). During matrix remodelling they may release pro-angiogenic growth factors including VEGF, TGF-β1, and basic fibroblast growth factor (bFGF) (P. Smith and Martinez 2006; Hughes 2008; Chun et al. 2007; Schoen and Mitchell 2013). Fibroblasts have been shown to play an important role in mediating epithelial cell morphogenesis and the formation of the complex dermal-epithelial junction, for example through modulation via adhesive molecules like fibronectin (Moharamzadeh et al. 2007; Mussig et al. 2008; Locke et al. 2008; Espinosa et al. 2010; Moharamzadeh et al. 2008).

The gingival lamina propria contains a dense vascular network. The nature of the vascular structures vary between the two anatomical components – the papillary and reticular component (Schroeder and Listgarten 1997). The papillary layer is directly below the epithelium.
and contains loosely organized collagen fibers and capillary loops, while the lower reticular layer contains bundles of collagen fibers (Dawson et al. 2014). The papillary component is dense with intraepithelial connective tissue papillae, and each papilla carries a terminal capillary loop. The average density of these clinically visible loops as determined using immunohistochemistry is 50-60 loops/mm². In addition, there is a vascular network with a plexus-like architecture, termed the gingival plexus. This basket-like network exists in a relatively small proportion, and is composed mostly of postcapillary venules (Figure 2-1B) (Schroeder and Listgarten 1997).

Periodontal disease, or periodontitis, is a chronic inflammatory disease that causes tooth loss in adults. Subgingival bacterial plaque pathogens initiate a host immune response leading to the destruction of the periodontal tissues that provide structural support for the teeth (Andrian et al. 2007). Periodontitis affects 47% of adults over the age of 30 (Eke et al. 2012). It is especially prevalent in the aging population – 70% of adults over 65 years of age suffer from periodontitis (Eke et al. 2012). Periodontitis is more severe in individuals with Type 1 and Type 2 diabetes, and has been found to have a role in cardiovascular disease progression (Southerland et al. 2006). Treatment of periodontitis requires the reduction or elimination of tissue inflammation induced by periodontal bacteria (e.g. via mechanical debridement of the tooth root and/or systemic antibiotic treatment) as well as the regeneration of the lost tooth-supporting structures (e.g. bone and soft tissue autografts and/or guided tissue regeneration) (Jain et al. 2008).

In the early stages of periodontal disease, complex bacteria biofilm communities colonize the regions between the tooth surfaces and the gingiva. They are able to accumulate due to architectural changes in the gingiva, namely a decrease in gingival attachment and subsequent formation of the gingival pocket (Holt and Ebersole 2005). Investigators have defined bacterial complexes associated with gingival health, gingivitis, and periodontitis by identifying major bacterial species present in plaque pathogens in each periodontal health state. As plaque biofilms progress from a healthy to disease state, Gram-negative rods, fusiforms, filaments, spirilla, and spirochetes begin to predominate over the Gram-positive cocci and rods in healthy plaque biofilms (Kistler et al. 2013). A later stage complex, known as the “Red Complex”, is made up of *Tannerella forsythia*, *Porphyromonas gingivalis*, and *Treponema denticola*. These three species are particularly recognized as being critical in the progression of periodontal disease (Delima and Van Dyke 2003; Dumitrescu 2010). *P. gingivalis*, a Gram-negative and non-motile
rod bacterium, has been associated with severe periodontitis. As an obligate anaerobe, it thrives in anaerobic environments such as the subgingival space. It produces adhesins, hemin-binding proteins, and proteinases that allow it to effectively colonize and exhibit virulence (Lamont and Jenkinson 2000). It is considered to be an endogenous species with an indeterminate latency period (Lamont and Jenkinson 2000).

Gingival recession is an additional periodontal pathology that can occur due to bacteria-associated periodontal disease or other health factors independent of oral hygiene including trauma, gender, tobacco consumption, or vigorous tooth brushing (Bottino et al. 2012). More than half of the population has one or more sites of pronounced gingival recession (Kassab and Cohen 2013). Autologous grafts, taken from the palate or buccal mucosa, are commonly used to repair gingival tissue defects (Figure 2-2). This surgical procedure has several drawbacks, including increased pain and morbidity at the donor site, potential tissue shortage, and the risk of a loss of original tissue characteristics upon transplantation (Moharamzadeh et al. 2007). Commercial alternatives include epithelial sheets and dermal grafts. Commercially available epithelial sheets are thin, difficult to handle and likely to contract upon implantation. Dermal grafts have not been clinically proven to increase gingival attachment; they can only be used to increase gingival thickness (Moharamzadeh et al. 2007).

Figure 2-2. Gingival grafting procedure. (A) Pre-op recession. (B) Flap elevated. (C) Subconnective tissue graft harvested from palate and sutured to recipient site. (D) Coronally advanced flap positioned and sutured. (From Jhaveri et al., 2010).
2.1.2 Current gingival tissue engineering strategies

Gingival tissue engineering is a periodontal regeneration strategy that has the potential to address the limitations associated with current clinical approaches for gingival tissue wound healing and regeneration. Tissue engineering can be used to generate full thickness oral mucosa constructs that supply the necessary cells as well as biochemical cues for the regeneration of the lamina propria and overlaying epithelium. These constructs consist of a three dimensional scaffold seeded with autologous cells, namely fibroblasts, producing ECM, promoting angiogenesis, and mediating epithelialization (Cheung, Rose, and Santerre 2013; Moharamzadeh et al. 2007, 2012).

The scaffold is an important aspect of engineered tissue. Tissue engineering scaffolds guide and localize cell growth while enhancing cell survival. They must be biocompatible so that they do not illicit a fibrotic response upon implantation, and they must degrade over time to allow the regenerated tissue to take over the defect space. Biomaterials used for gingival tissue engineering scaffolds may be naturally derived, synthetic, or a hybrid of both natural and synthetic biomaterials. Scaffolds made from natural ECM components such as collagen, fibrin, and collagen-derived gelatin are commonly used natural scaffold materials (McGuire et al. 2011; Mohammadi, Mofid, and Shokrgozar 2011; Köseoğlu et al. 2013; Rouabhia and Allaire 2010; Mohammadi, Shokrgozar, and Mofid 2007; Aroca et al. 2013; Mathes et al. 2010), however these scaffolds have inferior mechanical properties and an unpredictable and fast biodegradation process (Moharamzadeh et al. 2008; Cheung, Rose, and Santerre 2013). Decellularized dermis has also been used to create engineered oral mucosa equivalents (Yoshizawa et al. 2012; Izumi et al. 2003); however, these expensive scaffolds have unpredictable physical characteristics and limited fibroblast infiltration and migration (Moharamzadeh et al. 2008).

Synthetic scaffolds offer superior mechanical properties, reproducibility, and economic feasibility. Polyglycolic acid (PGA) was used as a scaffold in an early gingival tissue engineering study evaluated in a mouse model (Buurma et al. 1999), and is the basis for the commercially available dermal substitute sometimes used in the clinic for increasing gingival attachment (McGuire and Nunn 2005). More recently, PLGA, which has a slower degradation profile that is more suitable for tissue engineering applications, has been used as a scaffold material for engineered oral mucosa (Selim et al. 2011; Blackwood et al. 2008). In these studies,
PLGA was electrospun to form a non-woven fibrous mat. While the compatibility of synthetic polymers like PLGA with manufacturing methods such as electrospinning make synthetics an attractive option for scaffold materials, PLGA fails to match the biological compatibility of natural scaffold materials. When PLGA was evaluated against collagen and other natural-based scaffold materials for oral mucosal tissue engineering applications, natural polymers were shown to preferentially improve epithelium morphogenesis (Moharamzadeh et al. 2008), as well as fibroblast attachment and proliferation (Hakki et al. 2013; Kriegebaum et al. 2012).

PLGA continues to be the most ubiquitous of all synthetic polymers used for tissue engineering scaffolds; however, other synthetics have the potential to allow for an improved cellular and tissue response relative to PLGA. A degradable, polar/hydrophobic/ionic polyurethane, D-PHI, was developed for use as a tissue engineering scaffold with enhanced biocompatibility and mechanical properties (McBane, Sharifpoor, et al. 2011). It is synthesized using lysine diisocyanate and polycarbonate (PCN). Lysine diisocyanate is a non-traditional diisocyanate that produces lysine as a degradation by-product, a naturally occurring amino acid, and the PCN component allows the scaffold to be degraded by hydrolysis into non-acidic by-products (Sharifpoor, Labow, and Santerre 2009). Porous scaffolds made with D-PHI have a complex surface chemistry combining both polar and non-polar domains, which is believed to account for its improved cell attachment and promotion of a wound-healing phenotype in macrophages differentiated from seeded monocytes (McBane, Battiston, et al. 2011; McBane, Ebadi, et al. 2011). Scaffolds made from D-PHI have an interconnected micro- and macro-porous network, important for the diffusion of nutrients, waste, and cells throughout the scaffold and for promoting angiogenesis (Madden et al. 2010; Yang et al. 2001).

Cheung et al. found that HGF seeded on D-PHI and submitted to perfusion culture exhibited enhanced cell growth and type I collagen production, and that production of the contractile protein α-smooth muscle actin (α-SMA) by HGF was attenuated in flow conditions compared to static culture. It was speculated that D-PHI surface chemistry played an important role in modulating the myofibroblast phenotype by controlling the transfer of mechanical forces via the focal adhesion protein β1-integrin (Cheung, McCulloch, and Santerre 2014). Cheung et al. further demonstrated the suitability of D-PHI as a scaffold for gingival tissue constructs by optimizing several culture parameters in a co-culture of HGF and HUVECs to enhance the pro-
angiogenic character of the constructs (i.e. growth medium composition and cell seeding ratio of HGF relative to HUVECs) (Cheung et al. 2015).

Successful clinical translation of tissue engineering strategies relies on the establishment of a cell and tissue compatible scaffold material that has appropriate mechanical and degradation properties and is economically practical. While PLGA has failed to produce gingival or other oral mucosal engineered substitutes that match the quality of those produced on collagen or other ECM-protein based scaffold materials, D-PHI has the potential to allow for the generation of gingival constructs that match the quality of constructs produced using natural polymer materials, in part due to its unique surface chemistry that may help modulate myofibroblast differentiation and produce a pro-angiogenic character in constructs made up of HUVEC/HGF co-cultures.

2.2 Anti-infective Tissue Engineering Scaffolds

2.2.1 Biomaterial-associated infection

A tissue engineering scaffold used in periodontal tissue repair will by nature be placed in a highly infective environment. Biomaterials implanted into an infective environment such as the oral cavity are subject to bacteria adherence and biofilm formation. Bacteria associated with biomaterial-related infections can lead to damage of the biomaterials itself or degradation of tissue surrounding the implant, as well as the resistance of the bacteria to host defences and systemic antibiotic therapy (Gristina and Naylor 1996). Regenerative approaches to treat periodontitis, namely guided tissue regeneration (GTR) with barrier membranes, frequently fail due to the prevalence of infection (Hessam Nowzari, Matian, and Slots 1995; Jørgen Slots, MacDonald, and Nowzari 1999). Tissue engineering approaches using synthetic scaffolds have also failed due to microbial contamination (Rasperini et al. 2015), and as such methods for fighting infection of an implanted tissue engineering scaffold are widely reported to be of value (Vaquette et al. 2018). The bacterial colonies that accumulate on the biomaterial surface secrete extracellular polymeric substances (EPS) which form a matrix surrounding the bacterial cells. This matrix is defined as a biofilm, and is believed to be a key contributing factor to the persistence of biomaterial-associated infection (Gristina and Naylor 1996). The host response to the bacterial biofilm as well as to the plaque-associated with the latter, and periodontal wound
bacteria could potentially prevent healthy tissue regeneration in biomaterial-based strategies for gingival tissue engineering.

In the case of bacterial infection at an implant and/or wound site, neutrophils, macrophages, and lymphocytes persist in the environment. Phagocytic enzymes released by the inflammatory cells begin to damage the surrounding tissue (Costerton, Stewart, and Greenberg 1999). For example, elastase released by neutrophils for the degradation of microbiological components can degrade host ECM components including elastin, fibronectin, and collagen (Dumitrescu 2010). The persistence of these inflammatory cells during infection will stall wound healing as well as exacerbate tissue destruction at the implant site. Along with the host’s own immune cells, bacterial plaque pathogens also produce harmful proteins and enzymes that could lead to connective tissue degradation at the implant site (Dumitrescu 2010). *T. denticola*, one of the main etiological agents of periodontitis, has been shown to produce enzymes that degrade hyaluronic acid, fibronectin, laminin, and fibrinogen (Ellen and Galimanas 2005).

Investigators have characterized the microbiota associated with infection of barrier membranes, particularly expanded polytetrafluoroethylene (PTFE) membranes used in GTR regeneration techniques of periodontal tissues. Several investigators have demonstrated an inverse relationship between microbial counts on PTFE membranes and clinical gain attachment for the treatment of periodontal lesions (Hessam Nowzari, Matian, and Slots 1995; De Sanctis, Zucchelli, and Clauser 1996; Yoshinari et al. 1998; Selvig et al. 1992). *Streptococcus mutans*, the primary etiological agent of dental caries, and *P. gingivalis* been shown to have the strongest attachment to PTFE membranes (Wang et al. 1994). *P. gingivalis* has been implicated as being particularly important in failing periodontal tissue regeneration (Hessam Nowzari and Slots 1994), although *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia* (formerly *Bacteroides forsythus*) and *Peptostreptococcus micros* have also been reported present in failing GTR treatments (Hessam Nowzari and Slots 1994). The bacteria that colonize PTFE membranes are purported to originate either from the saliva or from the periodontal lesion in which it is being inserted (Slots, MacDonald, and Nowzari 1999), and the colonization of membranes can start as early as the time of insertion (Nowzari et al. 1996). Colonization that occurs during insertion has been associated with a reduced clinical attachment gain (Nowzari et al. 1996).
Bacteria may colonize a biomaterial implant and lead to implant-associated infection if the host’s own cells do not first attach and integrate into the implant (i.e. “the race to the surface” (Gristina 1987; Zhao et al. 2014)). Vascularization of the implant is required for the natural host defenses to prevent infection of implanted materials. Inflammatory cytokines and chemokines in the local tissue, as a response of the implant’s direct inflammatory activity with respect to cells which migrate to the infection site via the vasculature and participate in the destruction of pathogens. Furthermore, the ECM contains natural antimicrobial molecules that can inhibit bacteria colonization (Brennan et al. 2006).

Biomaterial-associated infection is a more significant risk for tissue engineering applications in which a scaffold material, particularly a synthetic scaffold material, is implanted in a tissue which is continuously exposed to bacteria (e.g. skin, oral soft tissues), or tissues for which tissue integration and vascularization of an implant is expected to be slow (e.g. bone) (Nair et al. 2011). In one clinical study of a polycaprolactone (PCL) scaffold to repair a periodontal bone defect, wound dehiscence and exposure of the PCL scaffold led to subsequent microbial contamination and ultimate failure of the implant (Rasperini et al. 2015). Investigators have proposed the use of antimicrobial scaffolds for oral tissue regenerative strategies (bone and soft tissues) (Bottino et al. 2013; Bottino et al. 2014; Shi et al. 2011; Terada et al. 2012). The utility of incorporating antibiotics in synthetic GTR membranes has recently been validated in a rodent model (Mathew et al. 2017).

2.2.2 Antibiotic-loaded scaffolds

The clinical translation of engineered gingival tissues will require investigators to address the potentially complicating effects of infection on wound healing and tissue regeneration (Ekenseair, Kasper, and Mikos 2013). Along with periodontal tissue engineering, infection control has been suggested as a necessary step for successful regeneration in the clinic for other applications of tissue engineering such as bone regeneration (Shi et al. 2011; Spicer et al. 2012).

Polymer-based antimicrobial delivery systems have been explored as a means to deliver drug locally and in a controlled system. Local and controlled delivery addresses important drawbacks of the use of systemic antibiotics, including systemic toxicity and the risk of promoting antibiotic-resistant strains of bacteria. Although a high concentration of antibiotic at
the time of implantation is desired to prevent initial bacterial contamination of the scaffold, the concentration must not be so high as to cause a cytotoxic effect for the local host cell population. Furthermore, release must continue beyond this point at a concentration that is above the MIC of potential pathogens. Prolonged release of antibiotic below the MIC may favour the growth of antibiotic-resistant strains of bacteria and make any infection more difficult to treat.

In an early study, antibiotic release from PLGA discs and electrospun nanofibrous scaffolds showed prolonged release for one month and one week respectively (Yoo et al. 2004; K. Kim et al. 2004). Thermoreversible polymers such as pluronic F-127 have been studied for periodontitis applications as a means to deliver antibiotic matrices to the gingival pocket by injection (A. W. Smith 2005). Antibiotic loaded PLGA microparticles have also been incorporated into injectable collagen scaffolds to produce local drug delivery combined with collagen-enhanced tissue regeneration (Schlapp and Friess 2003). The latter study showed a release profile in which a high initial dose followed by a more sustained release (approximately one week) was achieved.

Transplantation of a tissue engineered construct for treating bone defects involves prolonged open surgery, and reduced blood flow and the formation of necrotic tissues and fluids present challenges in the host’s fight against infection (Nair et al. 2011). Chang et al. suggested that providing a tissue engineered construct with antibacterial activity before adequate vascularization has occurred would fight infection and improve construct compliance. They showed that alginate beads loaded with vancomycin had no impact on the osteogenic capability of engineered bone constructs consisting of a demineralized bone matrix and MSCs (Chang et al. 2013). Xing et al. designed an infected bone defect model in rabbit tibias (Xing et al. 2012). They implanted a bone construct consisting of a fibrin scaffold and MSCs along with vancomycin alginate beads and found that the constructs exhibited more osteogenic potential compared to those scaffolds without the vancomycin beads. Spicer et al. investigated the effect of antibiotic release from colistin-loaded alginate and PLGA microparticles incorporated into porous polymethylmethacrylate (PMMA) space maintainers used in craniofacial surgeries (Spicer et al. 2013). Extended release of locally delivered antibiotic via the PLGA microparticles showed greater soft tissue healing when compared with implants that showed a quick burst release of antibiotic.
For soft tissue engineering applications, electrospinning blends of polymers and antibiotics is a particularly attractive approach for fabricating anti-infective scaffolds for tissue regeneration, as antibiotics and polymers can be blended and electrospun into highly porous mats in a straightforward manner. However, poor antibiotic-polymer physical interactions leads to a burst release of antibiotics that are incorporated directly into the polymer (Rambhia and Ma 2015). This excludes their usefulness for longer-term applications. The rate of drug release has been shown to vary with different antibiotics ((Gilchrist et al. 2013b; Toncheva et al. 2012)), the form in which a certain antibiotic is loaded in the fibres (acid, base, salt form) ((Gilchrist et al. 2013b; Zeng et al. 2005)), the drug loading ((Cui et al. 2006; Gilchrist et al. 2013b; Natu, de Sousa, and Gil 2010; Peng et al. 2008; Weldon et al. 2012)), as well as with the addition of additives such as PEG (Toncheva et al. 2012; K. Kim et al. 2004). Coaxial electrospinning, emulsion electrospinning and drug-loaded nanoparticle additives have all been used in an effort to slow down the release of drug molecules from electrospun scaffolds (Ignatova et al. 2013). However, it must be noted that a shortcoming of these studies is that non have investigated the propensity of released drug to cause antibiotic resistance in strains cultured with released antibiotic or other release products.

Polymer-based drug delivery systems may be designed such that drug is delivered at a controlled dose and time, triggered via external cues such as pH or temperature (Qiu and Park 2001). A polyurethane material that releases fluoroquinolone antibacterial agents from its backbone as it is degraded by the enzymes generated from macrophages was proposed as a “smart” release polymer that releases drug in proportion to the extent of bacterial infection at the implant site (M. Yang and Santerre 2001; Woo et al. 2002). Upon implantation, the immune response produces the necessary enzymes for degradation of the implanted material, which leads to the release of drug that prevents the formation of biofilm and an associated infection. Once healing has progressed, the level of enzyme production decreases and thus drug release is minimized. However, biodegradation and antimicrobial efficacy studies revealed the release of inactive drug still bound to polymer fragments (Woo, Mittelman, and Santerre 2000). Furthermore, the drugs had poor reaction kinetics, leading to the formation of short polymer chains (M. Yang and Santerre 2001).
Partly to address these limitations, a novel oligomeric monomer containing the fluoroquinolone antibiotic ciprofloxacin was developed recently for application in dental resins for the prevention of secondary caries (Delaviz, Liu, et al. 2018; Delaviz, Nascimento, et al. 2018). The CF that is released via hydrolysis is covalently linked to the polymer backbone as a pendant molecule such that the bioactive biodegradation by-products are limited to ciprofloxacin or ciprofloxacin/polyethylene glycol (PEG). However, due to the complexity of the oral microenvironment as well as the difficulty in closely controlling drug release from a polymer-based device, further mechanisms are required to ensure biological conditions associated with antibiotic use do not pose a risk of promoting the development of antibiotic resistance bacteria strains. Pathogens may become resistant to CF via mutations in the target sites (DNA gyrase and topoisomerase IV) or due to a decrease in cellular uptake of CF, associated with an increase in bacterial impermeability to the agent or the overexpression of efflux pumps (Ruiz 2003). Devices may incorporate combinations of two or more antibiotics with different modes of action such that the combination broadens the spectrum of activity in order to potentially reduce the likelihood antibiotic-resistance (Ashbaugh et al. 2016; Delaviz, Liu, et al. 2018). Alternatives to antibiotics may also be used as a means to fight infection in the oral environment, including surface modifications (chemical or topographical), incorporation of natural antibiotics, and use of heavy metals (Cattò, Villa, and Cappitelli 2018).

2.3 Prevascularization of Tissue Engineered Constructs

2.3.1 Angiogenesis and vasculogenesis

Tissue engineered constructs that exceed 200-400 µm in any dimension require a blood supply to ensure survival of implanted cells (Rouwkema, Rivron, and van Blitterswijk 2008; Auger, Gibot, and Lacroix 2013). This is because of the diffusion limitation of gases (oxygen, carbon dioxide) and nutrients and waste through tissues. Clinically relevant constructs for any tissue other than very thin skin tissue substitutes therefore require a means by which the engineered tissues are rapidly vascularized upon implantation. Angiogenic vascularization of the construct will take place during the wound healing response; however, this process can take days or weeks. Researchers in the field of tissue engineering are focusing on strategies to prevascularize full-thickness tissue engineered constructs as a means of accelerating angiogenic processes post-
implantation. A vascular network engineered in a construct *in vitro* can anastomose with the host vessels upon implantation to provide nutrients and oxygen to the cells within the construct (Kioke et al. 2004).

Engineering a prevascularized construct requires replicating significant elements of vasculogenesis and angiogenesis (the de novo formation of vessels and sprouting of new vessels from existing vessels, respectively) *in vitro*, so it is important to understand the underlying mechanisms behind these processes. Vasculogenesis occurs during embryonic development in the formation of the heart and the first primitive vascular plexus, or network, within the embryo (Semenza 2007). Mesodermal cells in the yolk sac called hemangioblasts differentiate to form angioblasts, endothelial precursor cells (Patan 2000). Angioblasts then differentiate into ECs, proliferate, and form the initial network. These processes rely on a threshold level of VEGF (Risau 1997). TGF-β has also been shown to be important for yolk sac vasculogenesis (Patan 2000). Vasculogenesis can also occur in adults, for example in tumor growth or profound tissue ischemia, in which case it is sometimes referred to as neovascularization rather than vasculogenesis.

In adult vasculogenic processes, bone-marrow derived endothelial progenitor cells (EPCs) circulate to the site of tissue growth via a coordinated sequence of signalling events, including adhesion, migration (by integrins), and growth factor, chemokine and cytokine signalling, produced as a result of physiological triggers (e.g. ischemia) (Hillen and Griffioen 2007; Schmidt, Brixius, and Bloch 2007). EPCs differentiate into ECs to form vascular networks in response to the secretion of pro-angiogenic growth factors by the recruited ECs. VEGF again is the most critical growth factor in this process, as well as angiopoietin and stromal cell-derived factor (SDF)-1α (Ribatti 2004). Placental growth factor (PLGF) has also been shown to be important (Ribatti 2004; Hillen and Griffioen 2007). Matrix metalloproteinases, specifically MMP-9, produced by recruited EPCs, allow for the release of factors important in promoting the proliferation of EPCs and their motility within the bone marrow (Ribatti 2004). Endothelial nitric oxide (eNO) was found to be important for the activation of MMP-9 by VEGF and SDF-1α (Aicher et al. 2003).
Angiogenic processes control vascular organization and remodelling, whether for embryonic development or for postnatal wound healing. These processes are distinguished from vasculogenesis and neovascularization in that they initiate the formation of new vessels from existing vessels, in contrast to the promotion of de novo network formation by differentiated progenitor cells that occurs in vasculogenesis. Hypoxia, physiological oxygen deficiency in tissue, is a major driver for angiogenic processes (Fraisl et al. 2009). Cells in hypoxic conditions produce pro-angiogenic growth factors and chemokines that regulate multi-faceted angiogenic signalling processes, directing ECs in local vessels to coordinate a spouting and branching action until the supply of oxygen is met and a quiescent phenotype can re-establish a new equilibrium state (Fraisl et al. 2009). Once capillaries are formed, further processes allow for these vessels to mature – diameter increases, the vessel wall becomes thicker, and mural cells (or pericytes) that surround the organized ECs proliferate and become contractile (Carmeliet and Jain 2011).

In post-capillary sprouting angiogenesis, the basement membrane around ECs in a pre-existing capillary or venule is degraded by MMPs. ECs form sprouts or “cords” along a provisional ECM that guides the EC directional sprouting via the actions of MMPs including MMP-2, -9, and -14 (Lokmic and Mitchell 2008). EC proliferation and organization into a lumen is influenced by pro-angiogenic growth factors VEGF, PLGF and FGF-2 (bFGF). Vessel maturation, including the necessary recruitment and differentiation of pericytes, is directed mainly by the growth factors platelet derived growth factor (PDGF)-β and angiopoietins (ANG-) (Lokmic and Mitchell 2008; Carmeliet and Jain 2011). These processes and their associated signalling molecules are outlined in greater detail in Figure 2-3. The molecular signalling that occurs in neovascularization and angiogenesis can be recapitulated and/or capitalized on in vitro in 3D scaffolds and co-cultures of ECs and supporting cells.
2.3.2 Prevascularization strategies

The natural lamina propria itself is a highly perfused tissue (Bullon et al. 2004); furthermore, cells implanted in a full-thickness construct that is not readily vascularized in vivo will become necrotic, and integration with the surrounding host tissue will be slow or non-existent (Auger, Gibot, and Lacroix 2013). Further still, implanted scaffold materials, particularly those which are

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**Figure 2-3. Molecular signalling in vessel branching (from Carmeliet and Jain 2011). Major events and associated molecular players are summarized.**

(a) **Selection of “tip cell”:** Basement membrane is degraded (MMPs), pericytes detach (ANG-2), and loosening of EC junctions (the vascular endothelial (VE)-cadherin adhesion molecules), promoted by VEGF. The tip cell is selected in the presence of VEGF receptors (e.g. VEGFR-2), neuropilins (NRPs), and the NOTCH signalling ligands DLL4 and JAGGED1. A signalling cascade induced by hypoxia is driven by hypoxia inducible factor (HIF)-1α. Tip cells have filopodia to sense environmental cues.

(b) **Stalk elongation and tip guidance:** The tip cell filopodias sense semaphoring, ephrin, protein signals and attach to ECM via integrins. Cells in the “stalk” proliferate and form a lumen in response to signalling molecules VEGF, VEGFR-1, NOTCH, WNT, placental growth factor (PLGF), and FGF. ECs associate via VE-cadherin and CD34. These stalk cells recruit pericytes via PDGF, ANG-1, NOTCH, and FGF.

(c) **Quiescent phalanx resolution:** Neighbouring branches fuse, and perfusion of the new lumen can commence which promotes a quiescent phenotype. EC junctions are re-established via production of VE-cadherin and ANG-1, pericyte maturation occurs (PDGF, ephrin, ANG-1, NOTCH, TGF-β1), and basement membrane is deposited via action of tissue inhibitors of metalloproteinase (TIMPs).
synthetic, may be prone to biofilm-associated infection once implanted in the infectious oral environment unless the natural host defense mechanisms can take effect as permitted only if perfusion of blood is present (Slots, MacDonald, and Nowzari 1999). Prevascularization of tissue constructs is an active area of research within the field of tissue engineering, whether for soft tissue (Frueh et al. 2017), muscle tissue (Kim, Hou, and Huang 2016), or for hard tissue applications (Santos and Reis 2010). Yet elucidating a successful prevascularization strategy remains one of the field’s major challenges (Laschke and Menger 2016).

Strategies for engineering microvessels in a biomaterial scaffold generally involve engineering a sophisticated scaffold material that is pro-angiogenic. This may be accomplished via the incorporation of geometric cues (Mirabella et al. 2017), mechanical cues (Y. C. Chen et al. 2012), or pro-angiogenic biomolecules such as growth factors (Amaral et al. 2018) and ECM proteins (Landau et al. 2017). It is also common to employ a co-culture technique, as ECs are widely reported to fail to organize or survive in a construct long-term without supporting cells such as fibroblasts, mesenchymal stem cells (MSCs) or other tissue-specific cells (Battiston et al. 2014).

Culturing the cell-seeded scaffolds under dynamic culture conditions, i.e. within a perfusion bioreactor, can activate shear-induced biochemical signalling pathways to promote EC invasion and organization (Kaunas, Kang, and Bayless 2011). Perfusion of growth medium also improves diffusion of gases (oxygen, carbon dioxide) and small molecules (glucose, amino acids, and waste products), and can guide angiogenesis by significantly affecting protein and macromolecule gradients within tissue matrices via convective forces (Shirure et al. 2017; Griffith and Swartz 2006). Hypoxic culture conditions may also be employed as a means of encouraging EC self-assembly via hypoxia-induced signalling pathways, though only a small number of studies have thoroughly investigated how oxygen gradients within the tissue matrix or scaffold affect angiogenesis (Phillips, Birnby, and Narendran 1995; Ottino et al. 2004).

Scaffolds that incorporate topographical or mechanical cues via micropatterning or 3D bioprinting of vessel-scale channels allow for close control over the engineered vessel geometry and density, and engineered microvessels with organized geometry have been shown to accelerate anastomosis with host vessels upon implantation in vivo (Baranski et al. 2013).
However, survival and patency of the implanted networks is not always maintained long-term (Mirabella et al. 2017; B. Zhang et al. 2016; Carmeliet and Jain 2011), and in some cases controlling geometry did not improve perfusion of the graft in vivo (Riemenschneider et al. 2016). It is worth noting as well that the controlled network geometries produced via micropatterning or 3D printing do not recapitulate the multi-directional microvessel networks present in vivo (Morgan et al. 2019), nor are the micropatterned scaffolds conducive to generating full-thickness grafts, as the vessel channels are organized in a planar manner. In addition, this biomaterial-based prevascularization strategy is limited as a translational strategy due to current issues associated with scaling and commercializing 3D bioprinters and bioreagents (Elomaa and Yang 2017).

While incorporating biochemical cues via embedded biomolecules can aid in methods that rely on EC self-assembly, this method also has drawbacks as a fully translational strategy. Peptides, growth factors, ECM proteins, and other commonly employed pro-angiogenic bioactive molecules are expensive and difficult to standardize. They also would limit the shelf-life of scaffolds used for tissue engineered constructs in the clinic. While the benefits of using a synthetic polymer scaffold versus a natural scaffold material are well recognized (Drury and Mooney 2003; O’Brien 2011), the majority of prevascularization strategies rely on the use of natural polymer materials as a starting point – in particular fibrin or collagen, materials that are both inherently conducive to EC invasion and tube formation. If a synthetic scaffold material is used, i.e. PLGA, then scaffolds are typically pre-treated with fibronectin (Santos et al. 2009; Bertlein et al. 2018), or fibrin is used as a cell-carrier during seeding (Freiman et al. 2018).

A summary of recent studies that use a non-patterned biomaterial-based approach to prevascularization are outlined in Table 2-1. The approaches used in these studies were tested in a rat or mouse in vivo model. Studies in which a synthetic scaffold was used are highlighted; however, as mentioned above, the studies rely on the use of bioactive adjuncts. It is evident that when a synthetic biomaterial such as PLGA is used as the scaffold material, cells are commonly seeded in a natural gel such as fibrin or collagen. This approach inherently increases variability in the seeding protocol and therefore the variability of the construct performance itself.
Table 2-1. State of the art summary: Select recent (2015-present) biomaterial-based strategies for non-patterned prevascularized constructs tested *in vivo* small animal.

<table>
<thead>
<tr>
<th>Biomaterial scaffold</th>
<th>Cells</th>
<th>In vitro Culture</th>
<th>Performance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLLA/PLGA</td>
<td>Tri-culture in fibrin • Human myoblasts • Genetically modified hECs secreting ANGPT1 • Genetically modified hSMCs secreting VEGF</td>
<td>Static; 4 days</td>
<td>• Functional host vasculature in implanted grafts was significantly greater compared to controls with non-modified ECs. • Engineered vessels had regressed almost completed by day 14 <em>in vivo</em>.</td>
<td>(Perry et al. 2018)</td>
</tr>
<tr>
<td>PLLA/PLGA coated with tropoelastin</td>
<td>Co-culture • Human AMECs and MSCs</td>
<td>Static; 24 hours vs. 14 days</td>
<td>• Covalently bound tropoelastin graft (CBT) supports vascularization and vessel maturation of PLLA/PLGA scaffolds <em>in vitro</em>. • 14-day CBT graft was almost completely vascularized within 12 days <em>in vivo</em>. The 24h CBT graft behaved similarly to a 14-day control. • No indication of what fraction of vessels were human origin.</td>
<td>(Landau et al. 2017)</td>
</tr>
<tr>
<td>3D printed poly(propylene fumarate) coated with fibrin</td>
<td>Co-culture • HUVECs • Human MSCs</td>
<td>Static; 1, 2 or 3 weeks</td>
<td>• Spheroids in the pores of the 3D-printed synthetic scaffold act an angiogenic sprouting points. • After 9 days <em>in vivo</em>, grafts cultured for 3 weeks <em>in vitro</em> had significantly greater vessel density than those with shorter <em>in vitro</em> culture periods. • Co-localization of perfusion and human-origin vessels were shown <em>in vivo</em> although the fraction of perfused engineered vessels was not quantified.</td>
<td>(Mishra et al. 2016)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Co-culture in fibrin/collagen gel • HUVECs • Human MSCs</td>
<td>Static, 24 hours</td>
<td>• hMSCs co-localized with HUVECs indicating they had taken a pericyte-like role. • Perfused engineered vessels in the graft were imaged up to 8 weeks <em>in vivo</em>. • Circulation velocity of red blood cells in the engineered vessels within the graft was quantified at 8 weeks and showed up to 4x slower velocity than host vessels.</td>
<td>(Pang et al. 2015)</td>
</tr>
</tbody>
</table>
The current state of the art, as highlighted in Table 2-1, also reveals that perfusion culture is not used regularly as a means of promoting in vitro prevascularization in random-pore scaffolds. Perfusion culture is more commonly used in microfluidic devices with micropatterned channels lined with ECs. The more common use of perfusion in such systems is possibly related to the investigators’ better control of shear stresses on cells in the channels, as shear is more readily estimated and standardized in controlled geometries (Z. Li and Cui 2014). Perfusion culture may be an important factor in promoting prevascularization of a random-pore scaffold construct that has thus far remained poorly studied.

Generally a longer in vitro culture time is beneficial (Mishra et al. 2016; Landau et al. 2017), though some investigators have used a pre-culture time as short as 24 hours with success (Pang et al. 2015). The studies highlighted in Table 2-1 report that engineered vessels typically regress over time or remain non-perfused once implanted in vivo. Therefore, a method to generate engineered vessels in synthetic scaffolds that have been shown to retain long-term functionality has yet to be described in the literature. Further, the value of providing a longer pre-culture period with perfusion flow may be beneficial in terms of establishing important tissue maintenance bio-factors for enabling successful grafting.

The functionality of tissue engineered microvessels has been determined by testing the perfusability and barrier function of the organized, CD31+ ECs, typically in an in vivo murine model (Rouwkema and Khademhosseini 2016). There are a range of established methods to test these parameters. Traditionally, end-point histology with immunohistochemistry of red blood cells and ECs can be used to quantify the number of perfused vessels in a tissue explant, although this method is limited in terms of determining barrier function (i.e. leakiness) of engineered vessels. It also fails to capture the 3D geometry of vessel networks (Tremblay et al. 2005). Intravital imaging using a window model (or in some cases directly through tissue (Tsiggou et al. 2010)) allows the investigator to more accurately capture the in vivo conditions around engineered vessels in terms of the extent of perfusion, leakiness, and geometry (Kioke et al. 2004). Most commonly, a fluorescently tagged blood pooling agent such as lectin or dextran is injected intravenously in the mouse prior to imaging with confocal or two-photon microscopy. Doppler perfusion (Mirabella et al. 2017; W. Zhang et al. 2015) and high resolution ultrasound
imaging techniques (X. Cai et al. 2013) have also been used for intravital perfusion characterization although to a lesser extent. A more comprehensive means of assessing vessel functionality includes a measurement of the blood velocity in the engineered vessels (Shandalov et al. 2014; Pang et al. 2015).

2.4 Summary

To combat the highly infective oral environment, a gingival tissue engineering construct must be able to facilitate tissue integration and vascularization in advance of bacteria attachment and biofilm formation of the scaffold material used. In this race to the surface, it is posited that the construct may combat bacteria attachment via incorporation of antibiotics into the scaffold for local delivery at the implant site. Antibiotic-loaded scaffolds for soft tissue engineering are typically electrospun polymers blended with an antibiotic. However, a burst release of antibiotic is common due to polymer/drug chemical incompatibility, which could lead to local tissue toxicity and/or subsequent release of antibiotic below the target MIC. Polymers and oligomers with covalently bound antibiotic have been fabricated such that the release of antibiotic is proportional to the amount of hydrolytic degradation of the macromolecule. In this way, antibiotic release is no longer dependent on diffusion-based processes alone, but rather is also dependent on more complex physiological processes such as degree of inflammation at the implant site.

Alternatively, a scaffold may be pre-seeded with ECs in order to generate a vascularized construct that anastomoses quickly once implanted. In this way, healthy tissue and natural host defense mechanisms can prevent the colonization of the scaffold by bacteria. Elucidating a competent strategy for prevascularizing a tissue engineered construct is one of the greatest challenges in the field of tissue engineering. Approaches include the use of sophisticated pro-angiogenic scaffolds (i.e. micropatterning, growth factor loading), co-culture (pre-seeding and culturing in vitro with ECs and one or more supporting cell type), and perfusion culture which improves diffusion and imparts mechanical cues to seeded cells. Due to the practical limitations of micropatterned and/or bioactive scaffolds, a means of prevascularizing a construct without the use of ECM-derived proteins, growth factors, or micropatterning would be beneficial.
This review of the literature reveals that the success of tissue engineering strategies for application in the infectious oral environment will depend on establishing a scaffold platform with controlled geometry and chemistry and allows for tissue regeneration without interference from oral pathogenic bacteria.
Chapter 3

3 Electrospun Polyurethane Nanofibre Scaffolds with Ciprofloxacin Oligomer: Effect on Drug Release and Cell Attachment versus Free Drug

This chapter reports on the fabrication of anti-infective gingival tissue engineering scaffolds fabricated via blend electrospinning of PCNU and AO, a CF-based oligomeric additive. The AO was hypothesized to promote a more uniform distribution of CF so as to prevent a burst release profile of drug once the scaffold is implanted. The findings reported here are related to Objective 1 of the thesis. The potential for the scaffolds to act as anti-infective gingival tissue engineering scaffolds is validated.

Highlights:

- The AO promoted a more uniform distribution of drug in the scaffold fibres relative to free CF.
- Gingival fibroblasts seeded on scaffolds with AO showed high viability; however, the scaffolds with free CF showed improved attachment of cells relative to AO.
- AO was found to segregate towards the surface of the fibres. This resulted in a fast release of AO when incorporated at a higher concentration in the fibres.
- The AO hydrolyzed to release CF slowly and in a linear manner.
- Scaffolds with AO inhibited bacterial growth.

Abstract

An electrospun degradable polycarbonate urethane (PCNU) nanofibre scaffold loaded with antibiotic was investigated in terms of antibacterial efficacy and cell compatibility for potential use in gingival tissue engineering. Antimicrobial oligomer (AO), a compound which consists of two molecules of ciprofloxacin (CF) covalently bound via hydrolysable linkages to triethylene glycol (TEG), was incorporated via a one-step blend electrospinning process using a single solvent system at 7 and 15% w/w equivalent CF with respect to the PCNU. The oligomeric form of the drug was used to overcome the challenge of drug aggregation and burst release when antibiotics are incorporated as free drug. Electrospinning parameters were optimized to obtain scaffolds with similar alignment and fibre diameter to non-drug loaded fibres. AO that diffused from the fibres was hydrolysed to release CF slowly and in a linear manner over the duration of the study, whereas scaffolds with CF at the same concentration but in free form showed a burst release within 1 hour with no further release throughout the study duration. Human gingival fibroblast (HGF) adhesion and spreading was dependent on the concentration and form the CF was loaded (AO vs. free CF), which was attributed in part to differences in scaffold surface chemistry. Surface segregation of AO was quantified using surface-resolved x-ray photoelectron spectroscopy (XPS). These findings are encouraging and support further investigation for the use of AO as a means of attenuating the rapid release of drug loaded into nanofibres. The study also demonstrates through quantitative measures that drug additives have the potential to surface-locate without phase separating from the fibres, leading to fast dissolution and differential fibroblast cell attachment.
### 3.1 Introduction

Periodontal disease is a chronic inflammatory disease that causes sensitivity, root caries, and tooth loss in adults. Almost half of adults over the age of 30 are affected (Eke et al. 2012). Gingival recession occurs in adults regardless of oral hygiene, and is found in 58% of adults over 30 (Kassab and Cohen 2013). Autologous grafts taken from the palate are commonly used to repair gingival tissue defects due to periodontal disease or recession due to other factors. Gingival tissue engineering is a promising alternative strategy for the regeneration of soft tissue periodontium that has the potential to overcome several of the limitations associated with current regenerative therapies, including the increased pain and morbidity, as well as the potential tissue shortage associated with autologous grafts (Moharamzadeh et al. 2007). A tissue engineered gingival construct consists of a 3D scaffold seeded with fibroblasts which can be used to contribute to the reconstruction of the lamina propria and mediate epithelial cell morphogenesis (Taba et al. 2005; Giannopoulou and Cimasoni 1996; Moharamzadeh et al. 2012).

Electrospinning has been used extensively to fabricate tissue engineering scaffolds from natural and synthetic polymers, with potential applications in periodontal tissue regeneration (Bottino, Thomas, and Janowski 2011; Blackwood et al. 2008; Dan et al. 2014). Electrospun fibres can be oriented into an aligned morphology to control bulk mechanical properties and the cellular response. Fibre alignment has been shown to promote cell alignment, and may also yield a more desirable fibroblast phenotype by promoting the production of extracellular matrix (ECM) molecules (Johnson et al. 2006; Lee et al. 2005). A degradable polycarbonate urethane (PCNU) synthesized with a hard-segment component consisting of hexane diisocyanate (HDI) and butane diol, and a soft segment polycarbonate diol (PCN) ((Tang, Labow, and Santerre 2001a, 2001b)), has been electrospun into aligned nanofibres and coated with fibronectin to engineer connective tissues for spinal repair. The fibres formed a mechanically strong and elastic substrate for annulus fibrosis cell adhesion, phenotype maintenance and ECM accumulation while undergoing surface-mediated resorption (Yeganegi, Kandel, and Santerre 2010; Iu, Santerre, and Kandel 2014). The degradation by-products, which include CO2 and hydroxyl containing molecules, along with hexane diamine, showed good biocompatibility with AF cells at the rate of release present in the study. Consequently, this scaffold platform showed potential as a gingival tissue engineering scaffold material.
The application of electrospun PCNU nanofibre scaffolds in the infectious oral environment potentiates the risk of a biomaterial-associated infection. As such, the feasibility of integrating anti-infective functionality within the scaffolds has been explored. Antibiotic-loaded nanofibres can be readily formed by blend electrospinning polymer and drug mixtures. However, the burst release commonly observed when antibiotics are incorporated directly into the polymer excludes their usefulness for longer-term applications (Rambhia and Ma 2015). Coaxial electrospinning, emulsion electrospinning and drug-loaded nanoparticle additives have all been used in an effort to slow down the release of drug (Ignatova et al. 2013). Although core-shell fibres have been shown to enable more control over drug release, the strict arrangement of the electrospinning equipment in the coaxial method and the poor biocompatibility of emulsifiers in the emulsion method limit their biomedical applicability.

In the current work, an antimicrobial oligomer (AO) containing the antibiotic ciprofloxacin (CF) was provided in-kind and used to control antibiotic release from electrospun PCNU nanofibres fabricated via one-step blend electrospinning. The AO consists of two CF molecules covalently linked to triethylene glycol (TEG) via hydrolysable ester bonds (Figure 3-1). The AO/PCNU blend fibres were hypothesized to control drug release as the AO must be hydrolysed for the CF to have any bioactivity. The AO was anticipated to further control drug release by promoting a more uniform distribution of drug within the PCNU scaffold matrix by increasing the strength or extent of interactions between the added antibiotic and the PCNU and minimizing interactions between the CF molecules themselves, thereby improving the compatibility of the blend system. Hence, the goal of this study was to determine if the AO can successfully prevent aggregation of CF, and to assess how the concentration of AO in the PCNU affects drug release character, antibacterial activity and gingival cell compatibility of the PCNU nanofibre scaffolds.
3.2 Materials and Methods

3.2.1 Electrospun scaffold fabrication

Antimicrobial oligomer (AO) was produced and received in-kind from Interface Biologics Inc. (Toronto, Canada). AO is an oligomeric form of CF that hydrolyzes to form free CF at a controlled rate. PCNU was synthesized according to previously established methods with hexane diisocyanate (HDI, Sigma-Aldrich, Oakville, Canada) polyhexamethylene carbonate diol (PCN, Sigma-Aldrich) and butane diol (BD, Sigma-Aldrich) in a molar ratio of 3:2:1 (HDI:PCN:BD) (Tang, Labow, and Santerre 2001a). The PCN was degassed and dissolved in anhydrous N, N-dimethylacetamide (DMAC, EMD Millipore, Etobicoke, Canada) and then reacted with HDI in the presence of dibutyltin dilaurate (DBDL, Sigma-Aldrich, ~1x10⁻³ mol catalyst/mol NCO) for 4 hours at 60-70°C to form a prepolymer. BD was then added to carry out a chain extension which proceeded overnight at 60-70°C. The polymer was then precipitated in an ether/water solution (30% v/v) to wash the residual DBDL and unreacted prepolymer. The final polymer product was washed in water (5 x 3 hours) and dried under vacuum for 72 hours at 50°C. The polystyrene equivalent weight average molecular weight and polydispersity was determined using gel permeation chromatography (GPC) with a refractive index detector (40°C) and data acquisition software (Waters, Mississauga, Canada). The mobile phase was N,N-dimethylformamide (DMF; Sigma-Aldrich) with 0.05M LiBr, and columns were maintained at 80°C.
The PCNU and AO were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Aldrich) at concentrations corresponding to 0, 7 and 15% w/w equivalent CF with respect to the PCNU. A solution of PCNU and 15% w/w ciprofloxacin hydrochloride (free CF, Alfa Aesar, Ward Hill, MA, USA) was also prepared as a control. PCNU and free CF in HFIP formed a clear solution with no precipitate. The concentration of PCNU in HFIP ranged from 13.4-14.0% w/v. Concentrations were adjusted to maintain a similar solution viscosity between all samples, wherein the concentration of the solution was adjusted such that 0.1 mL of the polymer solution in a 1 mL syringe flowed through a 22-gauge needle in 64 ± 2 seconds under normal gravitational force. The conductivity of the electrospinning solutions was measured using a 2-cell conductivity probe (accurmet AB30, Fisher Scientific, New Hampshire, USA).

The solutions were injected at a rate of 0.5 mL/h onto a cylindrical mandrel rotating at 9.25 m/s or 18.90 m/s (for PCNU alone and PCNU with AO or free CF, respectively). The needle had a positive charge of 17.5 kV (for PCNU alone) or 17 kV (for PCNU with AO or free CF). An 18 kV voltage difference between the needle and collecting surface was maintained. Scaffolds were approximately 50 μm thick. Residual HFIP was removed from scaffolds by drying under vacuum at 50°C for 72 hours. A separate scaffold was electrospun for each experiment repeat (N=3).

3.2.2 Scaffold imaging

Scaffold fibre morphology was imaged using scanning electron microscopy (SEM). Scaffold sections were mounted onto steel stubs, sputter-coated to 10 nm with platinum using an SC515 SEC Coating Unit (Polaron Equipment, Uckfield, UK) and imaged using a Hitachi S2500 SEM (Hitachi, Mito City, Japan). Average fibre diameter and fibre alignment distribution were calculated using Image J image processing software (NIH, http://imagej.nih.gov/ij/). The widths of 15 fibres from each scaffold type were measured. The fibre alignment distribution was determined using the fast Fourier transform (FFT) processing technique in Image J (Blit et al. 2012). The variance filter was applied to a representative image from each scaffold type to highlight the fibre edges. The images were then transformed to graphical representations of their frequency domains using the FFT function to extract directional information. A summation of the pixel intensities along a straight line from the center of the FFT image for all 360° angles...
around the image was performed by using the Oval Profile plug-in (authored by Bill O’Connell, http://rsb.info.nih.gov/ij/plugins/oval-profile.html). Pixel intensity was then plotted from 0° to 180° to provide a graphical representation of the degree of fibre alignment.

The distribution of CF in the scaffolds and scaffold fibres was imaged using a Zeiss LSM700 confocal microscope (Advanced Optical Microscopy Facility, Toronto Medical Discovery Tower, Toronto, Canada). Dry scaffold sections were mounted onto glass slides behind cover slips using Krystalon Mounting Media (EMD Millipore). The objective was 40x/1.4 Oil and excitation was accomplished with a 405 nm laser. A collection bandwidth of 493-700 nm was used.

3.2.3 Gingival fibroblast compatibility assessment

A human gingival fibroblast cell line (HGF-1, ATCC CRL-2014) was used to provide a preliminary assessment of the cell compatibility of the electrospun scaffolds. Cells were cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; life technologies, Burlington, Canada) supplemented with 100 U/mL penicillin/100 mg of streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (Sigma-Aldrich). Scaffolds were sterilized by gamma-irradiation at a dose of 25 kGy (SOCAAR, University of Toronto, Toronto, Canada), and then loaded into CellCrown-96 scaffold holders (ScaffDex, Finland), and pre-conditioned with 25 μL DMEM at 37°C for 1 hour. HGF-1 were then seeded onto the scaffolds at 5000 cells per scaffold (25 μL of 50,000 cells/mL). Following 1 hour incubation at 37°C with 5% CO₂, 200 μL of additional DMEM was added before further incubation. After 24 hours, a live/dead (calcein AM/ethidium homodimer-1) stain was performed on 2 of each of the scaffold types (Molecular Probes, life technologies). Briefly, scaffolds were washed 3 times with phosphate buffered saline (PBS), incubated in live/dead stain prepared according to the manufacturer’s instructions for 20 minutes at 37°C, then rinsed 3 times with PBS. The cells were imaged using a Zeiss LSM700 confocal microscope (Advanced Optical Microscopy Facility), with a 10X (0.5 NA) lens. Excitation was performed using the 488 and 555 nm lasers with long pass 555 and 560 filters, respectively. Two more cell-seeded scaffolds were fixed in 3% glutaraldehyde (Sigma) in PBS for 24 hours, then dried in increasing concentrations of ethanol before undergoing critical point drying and sputter coating for SEM imaging. The experiment was repeated 5 times (n=10).
3.2.4 X-ray photoelectron spectroscopy (XPS) analysis

Elemental composition analysis of the scaffolds was performed by x-ray photoelectron spectroscopy (XPS) at take-off angles of 20°, 40° and 60° relative to the sample surface using the Theta-Probe XPS system (Thermo Scientific, Tewksbury, MA, USA) at Surface Interface Ontario, University of Toronto. Peak analysis was performed using Avantage Data System software (v. 4.78, Thermo Scientific).

3.2.5 In vitro drug release study

Sterilized scaffolds were punched into 20 mm diameter discs, weighed, placed in a 20 mL scintillation vial, and submerged in 3 mL of PBS at 37°C. Scaffolds in solution were flat and floating below the surface of the PBS. Separate and independent samples were incubated in a 37°C oven (humidity controlled) for 0, 1, 2, and 7 days, and each sample group was terminated at its pre-defined time point. Three separate samples were generated for each scaffold type and for each time point, and the experiment was repeated 3 times (n=9 for each scaffold type and each time point). All sample preparation was completed in a laminar flow hood using sterile techniques. At the end of the incubation period, the solution was withdrawn and stored at -20°C until further analysis. The scaffolds were then freeze-dried and stored at -20°C before being imaged using SEM or confocal microscopy. Sections of dried scaffold samples were dissolved in DMF with LiBr at 0.1 mg/mL and injected for GPC analysis and determination of molecular weight.

The incubation solution was analysed for AO and CF release using a Waters high performance liquid chromatography (HPLC) system with Waters 600 EF pump, a Kinetix Phenyl Hexyl column (Phenomenex, Torrance, CA, USA), and Waters 2996 photodiode array detector. A gradient method was developed to resolve the CF-related release products, using a binary mobile phase of acetonitrile with 0.1% v/v trifluoroacetic acid (TFA) and water with 0.1% v/v TFA. A 1.0 mL/min isocratic flow rate was employed. Calibration curves were obtained using solutions with 5, 10, 25, 50 and 100 μM AO or CF HCl. Each sample injection was 50 μL. Chromatograms used for peak area analysis were reported for 280 nm.
3.2.6 Antimicrobial activity

The periodontal pathogen *Porphyromonas gingivalis* (ATCC 33277, Manassas, VA, USA) was used to test the antimicrobial efficacy of the electrospun scaffolds. *P. gingivalis* was cultured anaerobically at 37°C in Todd Hewitt broth with 1% (w/v) yeast extract, 5% hemin, and 1% menadione (Sigma). Cultures were grown to mid-log (~14h after sub-culture) and diluted to ~10^6 colony forming units (CFU)/mL. 100 µL of suspension was then added to a solution of CF HCl in Milli-Q water serially diluted in water to create 100 µl solutions in a 96 well plate. A sterility control was prepared with 100µl of broth added to 100 µl of MilliQ water. Negative controls were prepared with 100µl of suspension added to 100 µl of MilliQ water. The plates were incubated at 37°C in an anaerobic environment for 48 hours; optical densities were read by a plate reader at 600 nm (Bio-Tek® FL600 microplate reader, Bio-Tek Instruments, Inc. Winooski, VT, USA). The minimum inhibitory concentration (MIC) was recorded as the minimum concentration for which the OD_{600} was not significantly different from the positive control. The experiment was repeated 3 times (n=3 samples per repeat).

In a separate experiment, scaffolds were punched into 5mm diameter discs and placed into wells of a 24 well plate (n=3 samples per scaffold type); 1 mL of 10^7 CFU/mL bacterial suspension was then added to each well. Bacterial growth at 0h, 24h and 48h was measured by plating serial dilutions onto agar plates with 5% defibrinated sheep’s blood (Fisher Scientific, Waltham, MA, USA), 5% hemin and 1% menadione. The plates were then incubated anaerobically at 37°C for 7 days and CFUs were counted. The experiment was repeated 3 times (n=9 per scaffold type).

3.2.7 Statistical analysis

Statistical analyses were performed using SPSS software (IBM, New York, version 22). Student’s t-test was used to determine the statistical significance of any differences between the mean values of two groups. In the case of more than two groups, one-way ANOVA was used. When statistical significance was found, post hoc analysis was used to determine which of the groups contributed to the statistical significance. Levene’s test of homogeneity of the variances was conducted. Tukey’s B was used in the case of equal variances. In the event that the variances were unequal, the unequal variance version of ANOVA (Welch) was used and Dunnett’s T3 was
applied for post hoc analysis. In all analyses, the significance threshold was set to $\alpha = 0.05$. Standard deviation (SD) or standard error (SE) is reported with all relevant data sets.

3.3 Results

3.3.1 AO promotes a uniform distribution of drug in electrospun nanofibres

PCNU was synthesized and the polystyrene-equivalent weight average molecular weight ($M_w$) was determined to be $1.08 \times 10^5$ g/mol, with a polydispersity index (PDI) of 1.56. The molecular weight and PDI were in the desired range for appropriate biodegradation characteristics (Yang et al. 2009; Yeganegi, Kandel, and Santerre 2010). The electrospun nanofibres showed alignment at 90° (Figure 3-2). Electrospinning parameters (mandrel speed of rotation and the electrical charge at the collecting surface) were modified in order to increase the degree of fibre alignment in scaffolds with AO or free CF, as the settings used to deposit fibres without drug could not produce sufficient fibre alignment in the scaffolds with drug. The decrease in fibre alignment was hypothesized to be related to an increase in the conductivity of the polymer solution. A factorial designed experiment was carried out to determine the relative effect of mandrel rotation speed and the charge at the injection needle and collecting surface on the degree of fibre alignment. An 18 kV voltage difference between the injection needle and the collecting surface was always maintained. A central composite design with three centre points was utilized. Increasing the rotation speed significantly increased fibre alignment ($p<0.05$), while increasing the negative charge at the collecting surface caused a slight but statistically insignificant increase in fibre alignment ($p<0.1$). The conductivity of the electrospinning solutions and the electrospinning parameters for each of the scaffolds are reported in Table 3-1. There was no significant difference in the average fibre diameter (~275 nm) between each scaffold type.
Figure 3-2. Scaffold Fibre Alignment. Pixel intensity plotted from 0° to 180° extracted from FFT images of fibres, representing the degree of fibre alignment for the 0% CF scaffolds (A), 7% w/w CF-AO scaffolds (B), 15% w/w CF-AO scaffolds (C), and 15% w/w free CF scaffolds (D).

Table 3-1. Scaffold Fabrication Conditions and Fibre Properties. Electrospinning solution conductivity and electrospinning parameters of each scaffold type.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Solution Conductivity (μS/cm)</th>
<th>Speed of Rotating Mandrel (m/s)</th>
<th>Charge at Needle, Mandrel (kV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% w/w CF</td>
<td>0.8</td>
<td>9.25</td>
<td>+17.5, -0.5</td>
</tr>
<tr>
<td>7% w/w CF-AO</td>
<td>14.7</td>
<td>18.90</td>
<td>+17.0, -1.0</td>
</tr>
<tr>
<td>15% w/w CF-AO</td>
<td>28.6</td>
<td>18.90</td>
<td>+17.0, -1.0</td>
</tr>
<tr>
<td>15% w/w free CF</td>
<td>7.3</td>
<td>18.90</td>
<td>+17.0, -1.0</td>
</tr>
</tbody>
</table>

The nanofibre morphology was examined using SEM (Figure 3-3A). Scaffolds with AO were free of gross defects while the 15% w/w free CF scaffolds had clumps outside the fibres that were believed to be aggregated CF. Furthermore, there was greater surface roughness visible on the fibres of scaffolds made with free CF than scaffolds generated using the AO (Figure 3-3A, inlay). This surface roughness was attributed to aggregated CF molecules, an effect that is more pronounced when CF is added in free form compared to when it is incorporated as part of
the AO. The fine surface features (~20x70 nm length x width) present on the 0% w/w CF and 7 and 15% w/w CF-AO scaffolds are believed to be an artifact of the titanium coating for SEM sample preparation. The fibres of the 0% w/w CF scaffold are inherently bundled together due to hydrogen bonding interactions; however, this effect was not present in the scaffolds with added drug.

Since CF is a fluoroquinolone antibiotic with fluorescent properties (Muchohi et al. 2011), it was possible to visualize the distribution of the AO and free CF within the scaffolds using confocal microscopy. Scaffolds with 15% w/w free CF showed aggregated fluorescent CF in the fibres and outside the fibres in clumps (Figure 3-3B). The aggregates were also visible under low magnification SEM of the 15% w/w free CF scaffolds, Figure 3-3A. No clumps or aggregates were evident in the scaffolds with AO.

Figure 3-3. (A) Scaffold Fibre Morphology. Representative low and high magnification (inlay) SEM images showing surface morphology. Scale bars are 50 µm and 600 nm (inlay). (B) Drug Distribution in Scaffolds. Confocal light microscopy images showing CF distribution in the scaffolds for the 0% CF scaffolds, 7% w/w CF-AO scaffolds, 15% w/w CF-AO scaffolds, and 15% w/w free CF scaffolds. White arrows indicate aggregated drug outside of fibres, while dashed lines circle drug aggregated on top or within fibres. Scale bars are 50 µm.
3.3.2 Fibroblast spreading depends on CF concentration and form in scaffolds

The compatibility of the antimicrobial scaffolds for use in future tissue engineering applications was evaluated using human gingival fibroblasts (HGF-1), the cells that are responsible for the maintenance and remodelling of the gingival connective tissue (Taba et al. 2005; Giannopoulou and Cimasoni 1996). Preliminary cell compatibility was assessed by examining the viability and morphology of HGF-1 cells attached to the scaffold surface. Representative live/dead stained images are shown in Figure 3-4A, and the corresponding percent viability and percent coverage is shown in Figure 3-4C. Although there was no significant difference amongst each scaffold type in terms of the viability of cells attached, there was significantly greater attachment and spreading on the scaffolds with free CF (Figure 3-4D). The cell morphology was further investigated using SEM (Figure 3-4B). Similarly to the live/dead stain images obtained via confocal, the SEM reveal that cells attached on the 15% w/w free CF scaffolds had a more spread, aligned morphology than those cells on the 0% w/w CF scaffolds or scaffolds with AO.
Figure 3-4. Viability and Coverage of HGF seeded on Electrospun Scaffolds. (A) Live/dead stained cells seeded scaffolds at 24 hours post-seeding. Live stain is calcein AM (green) and dead stain is ethidium homodimer-1 (red). All scale bars 200 μm. (B) Representative SEM images of cells seeded on the scaffolds at 24 hours post-seeding. Scale bars are 50 µm. (C) Percent viability and (D) percent coverage of cells attached to electrospun scaffolds at 24 hours post-seeding. Data are mean ± SD. Two images were taken per scaffold, and 2 scaffolds were used for each scaffold type for each experiment (experiment was repeated 3 times). *Viability was calculated only for scaffolds that had cells attached. ‡ significant difference (p<0.01).
3.3.3 AO is localized on the surface of the nanofibres

The release of antimicrobial containing molecules was sensitive to the concentration of AO loaded into the fibres. The cumulative release of AO throughout the period of study for the 7 and 15% w/w CF-AO scaffolds are reported in Figure 3-5. Both scaffolds showed a biphasic release pattern, characterized by a faster initial release in the first 24 hours followed by a much slower, sustained release. However, the 7% w/w CF-AO scaffolds had a slower and more modest release as compared to the 15% w/w CF-AO scaffolds (approximately 5 times less drug release rather than the anticipated 2 times less based on concentration differences in the matrix). It has been suggested that drug incorporated into polymer matrices at lower concentrations can be introduced more uniformly than drug at high concentrations, which becomes localized at the surface of the fibres due to charge localization during electrospinning (K. Kim et al. 2004; Natu, de Sousa, and Gil 2010). As such, it was postulated that the AO would preferentially locate on the surface of the 15% w/w CF-AO scaffold fibres relative to the 7% w/w CF-AO.

![Figure 3-5. Release of AO from Scaffolds. Cumulative release of AO in µg/mL and relative to the total theoretical loading based on the measured initial mass of each sample. Data are mean ± SD (n=9).](image)

In an effort to quantify the surface segregation of the incorporated AO and CF, surface-resolved XPS was used to measure the fluorine concentration in the scaffolds (fluorine is present in CF and not the PCNU). Atomic fluorine concentrations measured at varying incident angles relative to the surface revealed a greater than theoretical bulk concentration of fluorine on the
surface of the 7 and 15% w/w CF-AO fibres (Figure 3-6). A surface sensitive gradient of fluorine was evident for the scaffold fibres with 15% w/w CF-AO, suggesting a more exaggerated surface segregation of drug at the high concentration of AO when compared to the low concentration.

![Graph showing fluorine concentration on scaffold surfaces at varying penetration depths](image)

**Figure 3-6. At% of Fluorine Measured on Scaffold Surfaces at Varying Penetration Depths.** Surface fluorine concentration (at%) of the scaffold fibres at varying take-off angles (where 20° is the most surface sensitive, corresponding to ~3.4 nm penetration depth) compared to the theoretical concentration as calculated using statistical methods. * represents significant difference (p <0.05). Data are the mean ± SD (n=3 separate samples).

### 3.3.4 AO hydrolyses to release CF slowly

CF release studies were carried out in a simple chemical aqueous *in vitro* model. The cumulative release of CF is reported in **Figure 3-7**. AO was hydrolysed to release CF slowly and in a linear manner ($R^2 = 0.96$ and 0.98 for the 7 and 15% w/w CF-AO scaffold respectively) over a 7 day period, while 15% w/w free CF had a burst release of antibiotic at 100x the concentration of the 15% w/w CF-AO scaffolds (~500 µM after <1 hour incubation). No significant change in the molecular weight of the PCNU was detected over the period of incubation for all scaffold types.
3.3.5 Antimicrobial activity of scaffolds against *P. gingivalis*

In order to assess the activity of CF released from the scaffolds within the *in vitro* conditions of the current study, cultures of *P. gingivalis* were incubated with scaffold sections over a period of 48 hours and measuring the cell density over time. The 7% w/w CF-AO scaffolds did not release enough free CF within the conditions of the present experiment to affect bacteria cell growth (Figure 3-8). The 15% w/w CF-AO scaffolds released CF at an inhibitory concentration such that cell growth was halted for the duration of the study, while the 15% w/w free CF control scaffolds exhibited a bactericidal concentration of antibiotic resulting in cell death.

Figure 3-8. Survival Curve of *P. gingivalis* Exposed to Scaffolds. Log(CFU/mL) over time for cultures of *P. gingivalis* exposed to sections of electrospun scaffolds. Data are mean ± SD (n=9).
3.4 Discussion

Electrospun nanofibre scaffolds made from a PCNU have been evaluated for use as a scaffold platform for future applications in tissue engineering of the soft tissue periodontium. However, the use of a synthetic material in the infectious oral environment has the potential to lead to a biomaterial-associated infection, with periodontal pathogens delaying or inhibiting healing in periodontal tissue regeneration strategies despite the use of systemic and topical administration of antibiotics and antiseptics (Bottino et al. 2012). Antibiotics blended directly into electrospun scaffolds impose challenges due to an inevitable burst release of drug, leading to difficulty in optimizing dosing for longer-term applications (He, Huang, and Han 2009; Gilchrist et al. 2013b; Reise et al. 2012; Ruckh et al. 2012). Although a high concentration of antibiotic at the time of implantation is desired to prevent initial bacterial contamination of the scaffold, the concentration must not be so high as to cause a cytotoxic effect for the local host cell population, and release must continue beyond this point at a concentration that is above the minimum inhibitory concentration (MIC) of potential pathogens. The AO used in this study was hypothesized to promote a more sustained release of active CF from PCNU nanofibre scaffolds fabricated via blend electrospinning, as the oligomeric CF must be hydrolysed before it enables any bioactivity.

Within the field of electrospun antibiotic-loaded nanofibres, different types of antibiotics at different concentrations have been incorporated into nanofibres fabricated from a variety of polymers, and the rate of drug release has been shown to vary with different antibiotics (Gilchrist et al. 2013b; Toncheva et al. 2012), the form in which a certain antibiotic is loaded in the fibres (acid, base, salt form) (Gilchrist et al. 2013b; Zeng et al. 2005), the drug loading (Cui et al. 2006; Gilchrist et al. 2013b; Natu, de Sousa, and Gil 2010; Peng et al. 2008; Weldon et al. 2012), as well as with the addition of various additives such as polyethylene glycol (PEG) (Toncheva et al. 2012; K. Kim et al. 2004). However, each of these factors also have an effect on the fibre diameter and alignment, which inevitably influences the rate of diffusion-controlled drug release, as well as drug release dependent on matrix degradation, since the rate of degradation depends partly on fibre diameter as well (Dong et al. 2009). In the current study, while antibiotic concentration (7 vs. 15% w/w) and form (AO vs. free CF) were altered, the PCNU fibre diameter and alignment was maintained between all samples. This allowed for investigation of phenomena
other than fibre diameter and alignment that may affect drug release characteristics from nanofibre scaffolds, as well as cell attachment, an important parameter needed to demonstrate future applicability in tissue engineering applications.

Additives such as CF have the potential to alter fibre diameter by changing the viscosity of the polymer solution used for electrospinning. In the current study, the concentration of PCNU in the electrospinning solution was adapted for each scaffold type to maintain a similar solution viscosity for each electrospinning solution, wherein the solutions containing both PCNU and CF (free or AO form) had a higher concentration of PCNU when compared to solutions without antibiotic. The 0% w/w CF scaffolds had a greater distribution of fibre diameters, with some thicker fibres appearing for these scaffolds when compared to the scaffolds with AO or free CF (see Figure 3-3A). This same effect has been observed in other studies where CF was incorporated into electrospun polymer fibres (Unnithan et al. 2012). The AO and CF in the current study were added in their ionic salt form, and ionic molecules increase the charge density of the electrospinning solution, resulting in enhanced stability of the electrospun jet and higher elongation forces, yielding more uniform fibres (Zong et al. 2002; Lee, Kim, and Kim 2005).

The addition of ionic molecules increased the conductivity of the electrospinning solution (Table 3-1). The solutions with AO vs. free CF had different conductivities which may be explained by the different contributions of their respective counter ions, trifluoroacetic acid (TFA) and HCl (Zong et al. 2002). The conductivity of the electrospinning solution affected the alignment of the fibres – non-uniform electron charge on the surface of the solution jet can result in a more exaggerated whipping of the polymer stream (Shin et al. 2001; Xin et al. 2008). This effect was counteracted by increasing the speed of the rotating mandrel, which acts to pull the fibres into aligned formation. However, the extent of orientation using this method is limited, since a non-uniform charge accumulation on the deposited fibres interferes with the alignment of the fibres still being deposited. Increasing the negative charge at the collecting surface and decreasing the positive charge at the needle may have had a small contribution towards improving fibre alignment (although not statistically significant). However, perhaps a more effective approach would have been to decrease the charge of the electric field. In the current approach, the electric field was held constant at 1 kV/cm for all scaffolds. Interestingly, although the electrospinning solution with 15% w/w free CF had a lower conductivity than the solution.
with 15% w/w CF (AO) (7.3 vs. 28.6 μS/cm), the former scaffolds also had a lower degree of alignment (Figure 3-2C vs 3-1D). Hence, it was hypothesized that the chemical incompatibility of the CF with the PCNU caused localization of charges, further disrupting the interaction of the solution jet with the external field. The more uniformly distributed AO had a greater influence on the jet’s conductivity, but simultaneously had more uniform charge distribution in the solution jet.

In contrast to the scaffolds with free CF, which is known to aggregate in polymer nanofibres, the scaffolds generated with AO had smooth, defect-free fibres (Figure 3-3A), suggesting that the AO attenuated the tendency for CF to phase separate and aggregate. For the scaffolds with free CF, confocal microscopy revealed the existence of aggregated CF outside of the fibres in clumps as well as distributed non-uniformly within the fibres (Figure 3-3B). Polymer/drug chemical incompatibility likely resulted in the non-uniform distribution of CF in the scaffolds made with free CF due to phase separation under the high electric field that existed during electrospinning. For a polymer/drug mixture to form a single phase, the two components must be thermodynamically compatible, where compatibility refers to the ability of the two components to form a single phase. Marsac et al. found that the thermodynamic compatibility of small molecule pharmaceutical agents and synthetic polymers may be modeled based on the classical Flory-Huggins lattice theory for polymer solutions (Marsac, Shamblin, and Taylor 2006; Krause 1978). Incorporating CF as part of the AO which has a TEG spacer may have had the effect of increasing favourable PCNU/drug interactions due to the chemical influence of the TEG link which is often used as diluent agent to promote movement of polymer chains (Marsac, Shamblin, and Taylor 2006), thereby allowing for the production of fibres with more uniformly distributed drug. Since there tends to be an upper limit in the concentration of drug that can be loaded in electrospun polymer fibres due to the contributing factors of increased charge density, solvent solubility, and polymer compatibility (Gilchrist et al. 2013b), the CF in AO form appears to present a means of increasing the loading of an antibiotic in electrospun fibres while still maintaining uniform fibre morphology and drug distribution.

It has been reported that drug may localize on the surface of electrospun fibres due to the charge accumulation of the ionic drug molecules (K. Kim et al. 2004; Natu, de Sousa, and Gil 2010; He, Huang, and Han 2009). However, the actual extent of surface segregation has not been
thoroughly investigated, and hence in the current study XPS was used to quantify the amount of AO or CF surface segregation on the scaffold nanofibres. This study was completed to understand the difference in release between the low and high concentration of AO, as well as the differences in cell attachment on the scaffolds. While other researchers have more commonly attributed differences in release at different concentrations to changes in fibre diameter, the current study offers an alternate mechanism to consider when fibre diameters are kept relatively constant (Peng et al. 2008; Ruckh et al. 2012).

Atomic fluorine concentrations measured at varying surface depths revealed a greater than theoretical bulk concentration of fluorine on the surface of the 7 and 15% w/w CF-AO fibres, (Figure 3-6). A surface sensitive gradient of fluorine was evident for the 15% w/w CF-AO scaffold fibres, suggesting a more exaggerated surface segregation of drug at the high concentration of AO. The at% of fluorine at the surface of the 15% w/w free CF fibres was slightly lower than that of the 15% w/w CF-AO fibres, and no gradient was evident, likely due to the propensity of the free CF drug molecules to aggregate both within and outside the fibres of these scaffolds (see Figure 3-3). It can therefore be concluded that the CF molecules in the 15% w/w free CF scaffolds are also distributed non-uniformly in the z-direction. The concentration of fluorine present at the surface of the 15% w/w CF-AO fibres was similar to the theoretical concentration of fluorine in pure ciprofloxacin (~4.15 at%), suggesting that at the higher concentration, the CF molecules within the AO are preferentially located at the surface of the fibres such that there is no limit to diffusion. The extent of surface segregation for AO is evidently responsible for the faster and greater extent of release of AO from the 15% w/w CF-AO scaffolds as compared to the 7% w/w CF (AO) scaffolds.

HGF cells were able to attach to the scaffolds with 15% w/w free CF, and had comparable viability to cells attached to the 0% CF scaffolds and AO scaffolds. Furthermore, cells on the 15% w/w free CF scaffolds were more spread, showing greater overall coverage of the scaffold (Figure 3-4). It is hypothesized that surface segregation of the CF or AO lead to substantial differences in the surface chemistry for each scaffold, affecting protein and cell attachment to the scaffolds. The scaffolds with free CF would have presented a high number of carboxylic acid and amine functional groups, which are known to promote cell attachment and spreading (Faucheux et al. 2004), while the carboxylic acid of CF is not present in a high density
for the AO scaffolds, or scaffolds with no additive. Carboxylic acid groups have been shown to significantly promote the attachment of fibroblasts on both 2D surfaces ((Faucheux et al. 2004)) and electrospun nanofibres (Park et al. 2007). While multiple other factors may also be contributing to increased cell attachment and spreading on the free CF scaffolds (e.g. distribution of CF within PU, aromatic chemistry, increased carboxylates, stiffness of aggregates, and topographical cues due to the surface roughness of aggregated CF), the present results reiterate the finding that the AO and CF became surface segregated on the electrospun fibres, causing significant differences in surface chemistry between scaffolds with varying forms and concentration of drug and affecting not only drug release but also cell interactions.

The release of CF from the 7 and 15% w/w CF-AO scaffolds was dependent on both diffusion of AO from the scaffold and the hydrolysis of the AO. The AO was hydrolysed to slowly release CF over the duration of the study (Figure 3-7), so that while the scaffolds with 15% w/w free CF had released about 500 µM of free drug within the first hour of the study, the scaffolds with 15% w/w CF-AO had only released about 5 µM of free CF. By incorporating the CF as part of the AO, a more sustained release of CF is possible. The incremental release of CF at each time point for both the 7 and 15% w/w CF-AO scaffolds was above the MIC of CF determined for characteristic periodontal pathogen *P. gingivalis* (0.16-0.31µg/mL). *P. gingivalis* exposed to scaffold sections of the 15% w/w CF-AO and 15% w/w free CF scaffolds showed inhibited growth and complete bacteria cell death respectively (Figure 3-8). To this end, the difference in release between the 7 and 15% w/w CF-AO scaffolds demonstrates that the level of antimicrobial activity can be varied with concentration of the AO. Hence, this strategy may be effective in both preventing long term bacterial colonization of the scaffolds once implanted as well as initial deployment of bacteriocidal activity. However, the 15% w/w free CF scaffolds may not be able to provide long term resistance to bacterial colonization.

While the release of free CF from the 7 and 15% w/w CF-AO scaffolds was slow and sustained (Figure 3-7), the release of AO itself showed a biphasic release pattern, characterized by an initial rapid release followed by slower more sustained release (Figure 3-5). Natu et al. found a similar concentration dependent release profile for their polycaprolactone (PCL) fibres with timolol maleate and acetazolamide, in which total drug release for the lower concentration formulation was not achieved (approximately 50% released after 52 days), while high loadings
resulted in greater burst release and shorter periods of release (almost 90% released after just 2 days) (Natu, de Sousa, and Gil 2010). The burst release phase is attributed to drug dissolution from the surface, while the second phase is dependent on diffusion and/or polymer/AO degradation to allow for release of drug entrapped in the physically cross-linked and more crystalline regions of the polymer matrix. GPC was used to determine the extent of PCNU degradation over the 7 day period, which was nonexistent or minimal during the duration of the study. This may explain why release is slow during the second phase.

To the authors’ knowledge no other study has examined the effect of added antibiotic on electrospun scaffold cell attachment. This is likely because the strategy of using an electrospun scaffold for both tissue engineering and small drug delivery is not commonly conceived. The field of anti-infective scaffolds for tissue engineering is largely centred on bone tissue engineering with scaffold materials specific to hard tissue (Nair et al. 2011). Other groups have successfully shown cell attachment on electrospun scaffolds, but did not discuss the effect of drug form or type and drug loading on attachment (Xue et al. 2014). The current work emphasized the importance of utilizing the extensive body of knowledge from the literature, related to surface chemistry effects on cell attachment, when incorporating additives such as antibiotics into scaffolds designed for tissue integration.

The current investigation has provided insights into the chemical character of the new AO drug delivery platform technology. While future studies will demonstrate the applicability of the technology in a given in vivo model, the current characterization data allows for the generation of unique tissue engineering and drug delivery concepts, including the example formulation proposed in Figure 3-9. A “sandwich” structure is proposed wherein PU fibres with AO at a low to intermediate concentration (between ~7-15% w/w) comprise the middle of the sandwich so that over longer periods, as the fibres degrade, the drug would be available at later time points, and fibres with free CF (at varying concentrations) would be located on the top and bottom of the scaffold construct. Scaffolds generated in this fashion can therefore allow for cell attachment, as well as generate a high initial release of CF upon implantation, while still providing long term, incremental release of CF. Such a system may address critical needs in the regeneration of many tissues which interface with clean bacteria laden surfaces, including
gingival (Bottino et al. 2012), skin (Pillay et al. 2013), and corneal (Liu et al. 2013) tissue engineering.

Figure 3-9. Application of Sandwich-Structured Scaffolds for Gingival Tissue Engineering. A schematic of the potential application of AO and CF scaffolds as full thickness tissue engineered grafts for gingival tissue engineering.

3.5 Conclusion

Electrospun nanofibres with aligned fibre morphology and sustained release of CF were fabricated using free CF and AO containing CF. It was found that the rate of release of CF is dependent on the concentration and the form (AO vs. free) loaded into the fibres. The use of AO resulted in enhanced compatibility between the drug and the PCNU scaffold matrix, with the outcome that the phase separation and drug aggregation present in scaffolds with free CF was avoided. Surface characterization revealed a concentration-dependent surface segregation of AO that resulted in a rapid and greater extent of release for scaffolds at a higher concentration of AO, while release from scaffolds with the lower AO concentration may be dependent on the degradation of the scaffold’s polymer matrix to enhance diffusion. The scaffolds are proposed for further study and future use in gingival tissue engineering applications with local drug delivery for prevention of biomaterial-associated infection.

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**Disclosure of Conflict of Interest**

Author Dr. Paul Santerre is engaged as a senior contractual consultant for Interface Biologics Inc.
Chapter 4

4 Influence of Ciprofloxacin-based Additives on the Hydrolysis of Nanofibre Polyurethane Membranes

This chapter reports on the effect of the CF-based AO and CF on the longer term (>14 days) drug release of the electrospun PCNU scaffold, as well as the biodegradation character of the scaffolds. The conclusions reported here continue from Chapter 3 to address Objective 1. The potential for the scaffolds to act as anti-infective gingival tissue engineering scaffolds is further validated.

Highlights:

- The introduction of drug in either form (AO or CF HCl) was found to increase the hydrolytic stability of the electrospun degradable PCNU scaffold matrix.

- The alteration of hydrolysis kinetics was attributed to changes in the hydrogen bonding character and microstructure within the scaffolds, introduced by the presence of CF.

- Released CF from both the AO and CF HCl scaffolds had a similar minimum inhibitory concentration (MIC) to that of off-the-shelf CF.

Abstract

A degradable polycarbonate urethane (PCNU) and an antimicrobial oligomer (AO) were used to generate anti-infective nanofibre scaffolds via blend electrospinning. The AO consists of two molecules of ciprofloxacin (CF) bound via hydrolysable linkages to triethylene glycol (TEG). The membranes were conceived for use as tissue engineering scaffolds for the regeneration of soft tissues for the periodontium, where there would be a need for a local dose of antibiotic to the periodontal space as the scaffold degrades in order to prevent biomaterial-associated infection. Scaffolds were made using AO at 7 and 15% w/w equivalent CF and compared to scaffolds with 15% w/w CF (with HCl counterion). AO was hydrolysed and released CF continuously over 28 days, while the 15% w/w CF HCl scaffolds showed a burst release within hours, with no subsequent release in the subsequent 28 day period. Released CF from both the AO and CF HCl scaffolds had a similar minimum inhibitory concentration (MIC) to that of off-the-shelf CF. Interestingly, the introduction of drug in either form (AO or CF HCl) was found to increase the hydrolytic stability of the electrospun degradable PCNU scaffold matrix itself. The alteration of hydrolysis kinetics was attributed to changes in the hydrogen bonding character and microstructure within the scaffolds, introduced by the presence of CF. This study has revealed that in generating in situ drug release systems, the secondary effects of the added drug on the degradation properties of the polymeric carriers must be considered, particularly for systems that act dually as tissue engineering scaffolds.
4.1 Introduction

Electrospinning of polymers has been demonstrated as a facile and versatile method to generate bioactive polymer scaffolds with tailored architecture for a wide variety of applications, including tissue engineering and drug delivery (Sill and von Recum 2008). Using electrospinning, polymeric nanostructured fibre mats are produced via the charging of a droplet of polymer solution. The surface tension of the droplet is overcome by the electrostatic repulsions between like charges in the solution, leading to the rapid extrusion and elongation of thin polymer fibres which are accelerated towards an oppositely charged collecting surface. Antibiotics are amongst a large number of bioactive agents that have been incorporated into electrospun nanofibre scaffolds, commonly added to generate scaffolds for local release of drug to control infection. Researchers have explored the feasibility of manufacturing anti-infective electrospun scaffolds for the prevention of abdominal adhesion (Bolgen et al. 2006), wound healing (K. Kim et al. 2004), bone tissue engineering (Gilchrist et al. 2013a), and endodontic and periodontic therapy (Bottino et al. 2014; Bottino, Thomas, and Janowski 2011).

An important parameter in the design of a drug delivery or tissue engineering scaffold is the biodegradation rate of the scaffold matrix. The rate of biodegradation of drug-loaded scaffolds affects the rate of release of drug trapped within the scaffold matrix, and the rate of biodegradation of a tissue engineering scaffold must be aligned with new tissue formation to promote tissue integration. While it is recognized that the biodegradation rate is a key design parameter for developing drug-incorporated scaffolds, the field has devoted little attention to understanding the mechanism behind changes in biodegradation characteristics when introducing drugs into electrospun scaffolds, despite evidence that the addition of drugs have the potential to alter bulk polymer (Siegel et al. 2006), microparticle (Anderson and Shive 2012) and as well as nanofibre degradation rate (Cui et al. 2006; Huang et al. 2006). Differences in the degradation rate for microparticles or bulk polymer constructs caused by the addition of drug has been attributed to differences in wetting properties (Cui et al. 2006; Huang et al. 2006), and in some cases autocatalysis (Dong et al. 2009).

In the current work, a degradable polycarbonate urethane (PCNU) synthesized in the authors laboratory, and an antimicrobial oligomer (AO), provided in-kind from Interface
Biologics Inc, were used to generate ciprofloxacin (CF) containing aligned nanofibre scaffolds via blend electrospinning. The AO consists of two CF molecules covalently linked to triethylene glycol (TEG) via hydrolysable ester bonds (Supplemental Fig. 4S-1). The AO has the potential to promote a more sustained release of CF in comparison to CF loaded directly into the electrospun matrix, as the oligomeric CF must be hydrolysed before it exhibits any bioactivity. Furthermore, the AO interacts with the polyurethane as a polymer rather than a small molecule and therefore achieves a well blended distribution of the drug throughout the polymer (Wright et al. 2017). This has the result of potentially modulating the rate of drug diffusion from the scaffolds. Given the polar structures of CF and the PCNU polymer it was desired to test the hypothesis that the introduction of drug into the degradable polymer would affect the rate of hydrolysis by influencing the hydrogen bonding and microstructure character that has been shown to be important determinants of PCNU degradation (Tang, Labow, and Santerre 2001a, 2001b). As such, the goal of the current work was to determine the effect of the CF concentration and form (AO vs. CF HCl) on the hydrolytic degradation rate of the electrospun PCNU scaffolds.

4.2 Materials and Methods

4.2.1 Polymer preparation

Antimicrobial oligomer (AO) was received in-kind from Interface Biologics Inc. (Toronto, Canada). (AO structure and purity were confirmed by $^1$H-nuclear magnetic resonance (H-NMR) and high performance liquid chromatography.) The polycarbonate polyurethane (PCNU) was synthesized according to previously established methods with hexane diisocyanate (HDI, Sigma-Aldrich, Oakville, Canada) polyhexamethylene carbonate diol (PCN, Sigma-Aldrich) and butane diol (BD, Sigma-Aldrich) in a molar ratio of 3:2:1 (HDI:PCN:BD) (Tang, Labow, and Santerre 2001a). The PCN was degassed and dissolved in anhydrous N, N-dimethylacetamide (DMAC, EMD Millipore, Etobicoke, Canada) and then reacted with HDI in the presence of dibutyltin dilaurate catalyst (DBDL, Sigma-Aldrich, ~1x10$^{-3}$ mol catalyst/mol NCO) for 4 hours at 60-70°C to form a prepolymer. BD was then added and as a chain extender and the reaction proceeded overnight at 60-70°C. The polymer was precipitated in ether to extract DBDL and low molecular weight polymer. The final polymer product was washed in water (5 x 3 hours) and
dried under vacuum for 72 hours at 50°C. The polystyrene equivalent weight average molecular weight (M_w,PS) and polydispersity was determined using gel permeation chromatography (GPC) with a refractive index detector (40°C) and data acquisition software (Waters, Mississauga, Canada). The mobile phase was N,N-dimethylformamide (DMF; Sigma-Aldrich) with 0.05M LiBr, and the columns (Waters Styrage HR3, HR4 and HR5 in series) were maintained at 80°C.

4.2.2 Electrospun scaffold fabrication

The PCNU and AO were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Aldrich) at concentrations corresponding to 0, 7 and 15% w/w equivalent CF with respect to the PCNU. A solution of PCNU and 15% w/w ciprofloxacin hydrochloride (CF HCl, Alfa Aesar, Ward Hill, MA, USA) was also prepared. The concentration of PCNU in HFIP ranged from 13.4-14.0% w/v, to achieve a consistent viscosity.

As described in previous work (Wright et al. 2017), the solutions were injected at a rate of 0.5 mL/h from an 18-gauge blunt-tip needle onto a stainless steel cylindrical mandrel covered in aluminum foil rotating at 9.25 m/s or 18.90 m/s (for PCNU alone and PCNU with AO or CF, respectively). The mandrel is 6 cm wide and has a diameter of 16 cm. The needle had a positive charge of 17.5 kV (for PCNU alone) or 17 kV (for PCNU with AO or CF). An 18 kV voltage difference between the needle and collecting surface was maintained (1 kV/cm). Scaffolds were made to be approximately 50 µm thick. Residual HFIP was removed from the scaffolds by drying under vacuum at 50°C for 72 hours. A separate scaffold was electrospun for each experiment sample (N=3).

4.2.3 Scaffold imaging

Scaffold fibre morphology was imaged using scanning electron microscopy (SEM). Scaffold sections were mounted onto steel stubs, sputter-coated to 10 nm with platinum using an SC515 SEC Coating Unit (Polaron Equipment, Uckfield, UK) and imaged using a Hitachi S2500 scanning electron microscope (Hitachi, Mito City, Japan). Average fibre diameter and fibre alignment distribution were calculated using Image J image processing software (NIH, http://imagej.nih.gov/ij/). The widths of 15 randomly selected fibres from each scaffold type were measured. The fibre alignment distribution was determined using the fast Fourier transform.
(FFT) processing technique in Image J (Blit et al. 2012). Three representative images from each scaffold group were then transformed to graphical representations of their frequency domains using the FFT function to extract directional information. A summation of the pixel intensities along a straight line from the center of the FFT image for all 360° angles around the image was performed by using the Oval Profile plug-in (authored by Bill O’Connell, http://rsb.info.nih.gov/ij/plugins/oval-profile.html). Pixel intensity was then plotted from 0° to 180° to provide a graphical representation of the degree of fibre alignment. The fibre alignment parameter, $S$, can be calculated by the definition

$$ S = \frac{\sum_{\theta=1}^{180} F_\theta [3 \sin^2(\theta + \alpha) - 1]}{2 \sum_{\theta=1}^{180} F_\theta} \quad (1) $$

where $F_\theta$ is the alignment value at angle $\theta$ and $\alpha = 90° - \theta_p$, the image deviation of FFT alignment peak angle $\theta_p$ to 90° ($S=1$ for perfectly aligned fibres and $S=0$ for absolute random) (De Gennes and Prost 1993).

### 4.2.4 Water contact angle measurement

Water contact angle was measured to assess the effect of the AO or CF HCl on the scaffolds’ hydrophilicity and polar character. The static water contact angle was measured on the electrospun scaffold surface using a goniometer system (ramé-hart, Model 100, Mountain Lakes, NJ). Five drops of 0.5 μL MilliQ water were deposited per scaffold type ($n=5$). The contact angle was measured for the stable droplet.

### 4.2.5 In vitro hydrolytic degradation study

Scaffolds were sterilized by gamma-irradiation at a dose of 25 kGy (SOCAAR, University of Toronto, Toronto, Canada). Each sample was punched into a 20 mm diameter discs, weighed, placed in a 20 mL scintillation vial, and submerged in 3 mL of Dulbecco’s phosphate buffered saline (PBS) at 37°C (pH~7). Scaffolds in sterile filtered solutions were flat and floating below the surface of the PBS. Samples were incubated in a 37°C oven (humidity controlled) for 0, 1, 2, 7, 14 or 28 days. Three samples were made for each scaffold type and for each time point, and the experiment was repeated 3 times ($N=3$, yielding $n=9$ samples in total for each scaffold type and each time point). All sample preparation was completed in a laminar flow hood using sterile
techniques. At the end of the incubation period, the hydrolytic degradation solution was withdrawn and stored at -20°C until further analysis of hydrolysis products (e.g. chromatography, toxicity, anti-bacterial activity). The scaffolds were then freeze-dried and stored at -20°C before being imaged using SEM or confocal microscopy. Samples of the dried scaffolds were dissolved in DMF with LiBr at 0.1 mg/mL and injected for GPC analysis.

The hydrolytic degradation solution was analysed for AO and CF using a Waters high performance liquid chromatography (HPLC) system with Waters 600 EF pump, a Kinetix Phenyl Hexyl column (Phenomenex, Torrance, CA, USA), and Waters 2996 photodiode array detector. A gradient method was developed to resolve the CF-related release products, using a binary mobile phase of acetonitrile with 0.1% v/v trifluoroacetic acid (TFA) and water with 0.1% v/v TFA. A 1.0 mL/min isocratic flow rate was employed. Calibration curves for the AO and CF were obtained using solutions with 5, 10, 25, 50 and 100 μM AO or CF HCl. Each sample injection was 50 μL. Chromatograms were analysed at 280 nm. (Peaks corresponding to degradation products associated specifically with the PCNU were characterized previously (Tang, Labow, and Santerre 2003).)

4.2.6 Characterization of scaffold bulk chemistry via ATR-FTIR

Attenuated total reflectance Fourier transform IR spectroscopy (ATR-FTIR) was used to study the effect of the AO or CF HCl on the hydrogen bonding functional groups at the surface (~2 μm penetration depth) of the electrospun PCNU. Measurements were performed on 3 samples of each scaffold type, fabricated on different days, using a Perkin Elmer Spectrum One FTIR (Perkin Elmer, Shelton, CT, USA) with Universal ATR accessory with ZnSe/Diamond Crystal (Perkin Elmer) at the ANALEST lab facility (University of Toronto, Toronto, Canada). The scan range was 4000 down to 550 cm⁻¹ and averaged over 32 scans. Peak-fitting and integration was performed using GRAMS/AI spectroscopy software (Thermo Scientific, Tewksbury, MA, USA). Scaffolds from day 0 and day 28 of the degradation study were both analysed.

4.2.7 Characterization of scaffold microstructure via DSC

Differential scanning calorimetry (DSC) was used to assess changes in the scaffold microstructure due to the addition of AO or CF HCl. Each scaffold type was analysed using a TA
Instruments model 2910 differential scanning calorimeter at the Brockhouse Institute for Material Research (McMaster University, Hamilton, Canada). Approximately 5 mg of sample was used for each run. Thermograms were recorded between -100°C and 150°C at a heating rate of 20°C/min. Following heating to 150°C, samples were quenched to -100°C and run through a second heating cycle. A minimum of 2 samples were tested per type.

4.2.8 Determination of minimum inhibitory concentration of degradation release products

Periodontal pathogen *Porphyromonas gingivalis* (ATCC 33277, Manassas, VA, USA) was used to test the antimicrobial efficacy of the degradation products derived from the electrospun scaffolds. *P. gingivalis* was grown to a state of exponential growth at 37°C in an anaerobic chamber in Todd Hewitt broth with 1% (w/v) yeast extract, 5% hemin, and 1% menadione (Sigma). Culture suspensions were diluted to a concentration of 10^7 colony forming units (CFU)/mL in broth. Hydrolytic degradation and drug release products from day 28 of the degradation study were serially diluted in Milli-Q water to generate 100 µL test solutions in a 96 well plate. CF HCl in Milli-Q water was tested as a control. 100 µL of diluted culture suspension was then added to each test solution. A sterility control sample was also prepared with 100 µL of culture broth added to 100 µL of water. Positive controls with no bacteria were prepared with 100 µL of culture suspension added to 100 µL of water. The plates were incubated at 37°C in an anaerobic environment for 48 hours; optical densities were read by a plate reader at 600nm (Bio-Tek® FL600 microplate reader, Bio-Tek Instruments, Inc. Winooski, VT, USA). The minimum inhibitory concentration (MIC) was recorded as the minimum concentration for which the OD_600 was not significantly different from the sterility control after the 48h incubation period.

4.2.9 Gingival fibroblast compatibility assessment

A human gingival fibroblast cell line (HGF-1, ATCC CRL-2014) was used to test the cell compatibility of degradation and drug release products derived from the electrospun scaffolds. Cells were cultured at 37°C with 5% CO2 in Dulbecco's modified Eagle's medium (DMEM; life technologies, Burlington, Canada) supplemented with 100 U/mL penicillin/100 mg of streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (Sigma-Aldrich). HGF-1 were seeded onto tissue culture polystyrene 96-well plates using 200 µL of 50,000 cells/mL in DMEM per
well, and then incubated for 24 hours at 37°C with 5% CO₂ such that the wells were confluent with cells. The medium was then aspirated from each well and replaced with 200 μL sterile filtered solution containing degradation and drug release products obtained from the hydrolytic degradation study supplemented with DMEM powder (Life Technologies) and 10% fetal bovine serum. The solution used contained all degradation and drug release products accumulated after 7 days incubation of each scaffold type, since this time point had the highest release of drug products with a given time interval and would represent the strongest drug product dose. The solution was also diluted to 50% and 10% (v/v) degradation solution using medium comprised of DMEM powder in Dulbecco’s PBS with 10% fetal bovine serum, or FBS (to assess for dose response). DMEM in PBS with 10% FBS medium was also used as a negative control and supplemented with 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich) for the positive control. The cells were incubated in the test solutions for 24 hours at 37°C with 5% CO₂, and a metabolic activity and DNA assay were performed.

The metabolic activity was quantified using a WST-1 assay (Roche Diagnostics, Laval, QC, Canada). The test solutions were aspirated, and the cells were rinsed with 200 μL PBS. WST-1 reagent and DMEM were added to each well at a ratio of 1:10 WST-1:DMEM. The cells were then incubated for 1 hour at 37°C with 5% CO₂ and the absorbance was read with a spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA) at 450 nm using a reference wavelength of 650 nm. The metabolic activity of the cells incubated with each test solution is reported as the relative WST-1 absorbance compared to the negative control.

The DNA mass associated with number cells incubated with each test solution was measured using the CyQUANT assay (Life Technologies). Briefly, the cells in each well were rinsed with 200 μL PBS, and then stored at -70°C for a minimum of 3 hours to allow for cell lysis. After the addition of the CyQUANT reagent, the fluorescence was read using a fluorescence plate reader at an excitation wavelength of 480 nm and emission wavelength of 520 nm (Bio-Tek® FL600 microplate reader). DNA mass is also reported relative to the negative control.
4.2.10 Statistical analysis

Statistical analyses were performed using SPSS software (IBM, New York, version 22). Student’s t-test was used to determine the statistical significance of any differences between the mean values of two groups. In the case of more than two groups, one-way ANOVA was used. When statistical significance was found, post hoc analysis was used to determine which of the groups contributed to the statistical significance. Levine’s test of homogeneity of the variances was conducted. Tukey’s HSD was used in the case of equal variances. In the event that the variances were unequal, the unequal variance version of ANOVA (Welch) was used and Dunnett’s T3 was applied for post hoc analysis. In all analyses, the significance threshold was set to $\alpha = 0.05$. Unless otherwise stated, all experiments were repeated in triplicate (N=3) with n=3 samples per repeat (n=9 in total). Scaffolds fabricated on different days were used for each repeat.

4.3 Results

4.3.1 Fibre alignment and morphology

Polycarbonate polyurethane with a polystyrene equivalent weight average molecular weight ($M_{w,PS}$) of 9.94x10$^4$ g/mol and polydispersity index of 1.51 was electrospun into droplet-free nanofibres showing alignment at 90° (Figure 4-1). Electrospinning parameters were modified in order to increase the degree of fibre alignment in scaffolds with AO and CF HCl to offset the greater whipping instability caused by perturbations to the charge distribution within the polymer solution upon addition of drug. The speed of the rotating mandrel was increased from 9.25 m/s (no-drug sample) to 18.90 m/s (for CF containing samples) in order to accommodate the latter. The speed of the mandrel, the degree of fibre alignment and the average fibre diameter for each of the scaffolds are reported in Table 4-1. There is no significant difference between the average fibre diameters for each scaffold type. Under low magnification, fibre defects are visible in the 15% w/w CF HCl scaffolds, hypothesized to be clumps of aggregated CF (Supplemental Fig. 4S-2).
Figure 4-1. Drug-free, AO and CF scaffolds have similar fibre morphology. Representative SEM images of the 0% CF (A), 7% w/w CF (AO) (B), 15% w/w CF (AO) (C), and 15% w/w CF HCl (D) electrospun scaffolds. All scale bars 12 μm.

Table 4-1. Scaffold fibre alignment and diameter is quantified. Fibre alignment parameter, S (where S = 1 for perfectly aligned fibres and S = 0 for absolute random (De Gennes and Prost 1993)) and average fibre diameter of electrospun fibres (n=15 ± SD).

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Fibre Alignment, S</th>
<th>Average Fibre Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% w/w CF</td>
<td>0.648</td>
<td>274 ± 120</td>
</tr>
<tr>
<td>7% w/w CF (AO)</td>
<td>0.614</td>
<td>305 ± 86.5</td>
</tr>
<tr>
<td>15% w/w CF (AO)</td>
<td>0.694</td>
<td>251 ± 62.8</td>
</tr>
<tr>
<td>15% w/w CF HCl</td>
<td>0.428</td>
<td>307 ± 68.4</td>
</tr>
</tbody>
</table>

4.3.2 Water contact angle

It was found that there is a significant difference in the water contact angle between the 0, 7 and 15% CF (AO) scaffolds as well as the 15% w/w CF HCl scaffolds (109.5±1.9, 82.4±4.1, 67.2±8.8, and 64.4±7.5° respectively, n=5, mean ± SD, Supplemental Fig. 4S-3). Given the similarity in the fibre size and relative distribution (Figure 4-1), a lower water contact angle in the 15% w/w CF (AO) and CF HCl scaffolds indicates an increase in the hydrophilicity and wettability of these scaffolds which is directly associated with the polar chemistry of the CF molecules.
4.3.3 Hydrolytic degradation of polymeric scaffolds and drug release properties

The amount of CF released via AO hydrolysis during the 28-day in vitro degradation and drug release study is shown in Figure 4-2. The release of CF from the AO is compared to the release of CF from the 15% w/w CF HCl scaffolds. AO released from the scaffolds was hydrolysed to release CF slowly over the duration of the study, while the 15% w/w CF HCl had a burst release of antibiotic yielding a concentration 100x greater than that of the 15% w/w CF (AO) scaffold samples within the first hour of study. The extent of release for the AO scaffolds is sensitive to the concentration which it is loaded into the fibres. The total cumulative release of AO over 28 days in terms of percent of theoretical loading based on the measured initial mass of AO in each sample was 24.92 ± 9.79% and 90.89 ± 15.45% from the 7 and 15% w/w CF (AO) scaffolds respectively, while the 15% w/w CF HCl released 100% of the loaded CF (all within the first hour of the study).

Figure 4-2. CF release depends on concentration and form of drug loaded. Cumulative concentration of CF in micromolars (μM). Data are mean ± SD (n=9).

The extent of scaffold degradation was investigated using GPC and the percent $M_w,PS$ of the initial value (day 0 time point) was calculated (Figure 4-3). The PCNU scaffolds are sensitive to hydrolytic degradation via the urethane and carbonate groups (Tang, Labow, and Santerre 2001a). However, only the 0% w/w CF and 7% w/w CF (AO) scaffolds underwent a significant change in $M_w,PS$, suggesting that the added AO and CF HCl increased the hydrolytic
stability of the electrospun scaffolds. More importantly, the protective effect remained in place for the 28 day period despite the significant release of CF and AO for the latter samples.

Figure 4-3. Scaffolds with CF and AO showed decreased hydrolytic degradation. Reduction in polystyrene equivalent weight average molecular weight (M_w,PS), as a percent (%) of original (t=0 days), determined by GPC for the 0% w/w CF, 7% w/w CF (AO) scaffolds, 15% w/w CF (AO) scaffolds and 15% w/w CF HCl scaffolds. Data are the mean ± SD (n=9).

High magnification SEM images taken of the scaffold nanofibres following the 28 day degradation study reveal that the scaffold fibres fused together, particularly for the 0% w/w CF and 7% w/w CF (AO) scaffolds, the two scaffold types that underwent significant hydrolysis (Figure 4-4). The average fibre diameter before and after the degradation study is significantly different for all scaffold types, however this difference is most pronounced for the scaffolds that were shown to have undergone significant hydrolysis (Figure 4-5).
Figure 4-4. Degraded fibres: Fusing and an increase in fibre diameter is evident. Representative low and high magnification (inlay) SEM images of fibres from the (A) 0% w/w CF, (B) 7% w/w CF (AO), (C) 15% w/w CF (AO), and (D) 15% w/w CF HCl scaffolds at 28 d. Scale bars 12 µm and 1.2 µm (inlay).

Figure 4-5. Increase in fibre diameter is greatest for 0% w/w CF scaffolds. Average fibre diameter before and after the 28-day degradation study. Data are mean ± SD (n=9, 15 fibres measured per sample). There is a significant difference between as-made and 28 d for each scaffold type (t-test, p <0.05).
4.3.4 Scaffold hydrogen-bonding chemistry measured via ATR-FTIR

Changes in polyurethane hydrogen bonding (H-bonding) resulting post-addition of AO or CF were detected using ATR-FTIR analysis. The ratio of H-bonded to non-hydrogen bonded peak areas for the chemical groups of interest from the as-made scaffolds and at the end of the 28-day degradation study are shown in Figure 4-6 (spectra shown in Supplemental Fig. 4S-4). Characteristic peaks at 3324 and 3400 cm\(^{-1}\) are assigned to H-bonded and non-H-bonded NH groups, respectively. Peaks at 1720 and 1740 cm\(^{-1}\) are assigned to H-bonded and non-H-bonded carbonate C=O, respectively (Tang, Labow, and Santerre 2001a; McCloskey, Yip, and Santerre 2002). A peak for the H-bonded urethane C=O can be seen at 1685 cm\(^{-1}\); the non-H-bonded urethane C=O is assigned to the small peak at 1700 cm\(^{-1}\) (Tang, Labow, and Santerre 2001a; McCloskey, Yip, and Santerre 2002). A peak at 1624 cm\(^{-1}\) in the 7 and 15% w/w CF (AO) scaffolds is attributed to the aromatic C=C stretch in CF. The addition of CF in the form of CF HCl introduced a significant decrease in the number of H-bonded amines, while simultaneously increasing the proportion of H-bonded carbonate carbonyls. The addition of AO at the higher concentration introduced a significant increase in the proportion of H-bonded carbonate carbonyls into the polymer, however not to the same extent as the CF HCl. Post-incubation in PBS for 28 days, the 0% w/w CF scaffolds have a significantly lower number of H-bonded amines and urethane carbonyls when compared to all the drug containing polymers, however the levels of H-bonded amines and urethane carbonyls in the polymer remain relatively unchanged for the 15% w/w CF (AO) and CF HCl materials. The 15% w/w CF (AO) and CF HCl scaffolds no longer have a higher proportion of H-bonded carbonate carbonyls after incubation for 28 days, when compared to the other two materials.
Figure 4-6. H-bonding before and after degradation depends on the form and concentration of drug added. Ratio of H-bonded to non-H-bonded peak areas for scaffolds as-made (A) and at 28 d (B). * represents a significant difference between groups, ** denotes significantly different from all other groups (p<0.05) for a given chemical functional group at each time point. Data are the mean ± SD, n = 3 (A) or 9 (B).

4.3.5 Scaffold microstructure measured via DSC

Polyurethanes have a phase-segregated microstructure made up of the soft and hard segment domains, and the degree of phase separation is in part determined by intermolecular H-bonding (Lamba, Woodhouse, and Cooper 1998). By influencing intermolecular H-bonding, the antimicrobial oligomer has the potential to introduce changes in the scaffold microstructure.

Transition temperatures measured via DSC provide an indication of the extent of phase mixing in the PCNU and were used to determine whether the AO or CF HCl had an effect on the electrospun scaffold microstructure. This was of particular interest since PCNU phase separation and soft segment crystallinity have been shown to affect hydrolysis characteristics (Tang et al. 2002; Tang, Labow, and Santerre 2001a). The glass transition temperature increases very slightly upon the addition of the AO or CF HCl (Table 4-2, Supplemental Fig. 4S-5). The first melt transition temperature (T_m1) is attributed to the crystalline regions in the polyurethane’s PCN soft segment, while the second melt temperature (T_m2) is attributed to the crystalline regions in the urethane hard segment. The AO introduces an increase in T_m1, from 46.0°C to 51.3 and 50.6°C in
the 7 and 15% w/w CF (AO) scaffolds respectively. The addition of CF HCl causes an even
greater increase in $T_{m1}$ (46.0 to 63.8°C). The second melt transition, $T_{m2}$, becomes less
pronounced upon the addition of the AO or CF HCl (and is not detectable in the 7% w/w CF
(AO) spectrum).

**Table 4-2.** DSC data for as-made fibres shows CF and AO affect PCNU transition
temperatures. Transition temperatures and associated enthalpies from DSC thermograms
of the electrospun scaffolds. $T_g$ = glass transition temperature, $T_{m1}$ = first melt transition
temperature, $T_{m2}$ = second melt temperature, and $\Delta H$ are the corresponding change in
enthalpy associated with each melt transition.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>$T_g$ (°C)</th>
<th>$T_{m1}$ (°C)</th>
<th>$\Delta H_1$ (J/g)</th>
<th>$T_{m2}$ (°C)</th>
<th>$\Delta H_2$ (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% w/w CF</td>
<td>-36.9</td>
<td>46.0</td>
<td>3.74</td>
<td>103.0</td>
<td>14.8</td>
</tr>
<tr>
<td>7% w/w CF (AO)</td>
<td>-37.0</td>
<td>51.3</td>
<td>3.09</td>
<td>not detected</td>
<td>not detected</td>
</tr>
<tr>
<td>15% w/w CF (AO)</td>
<td>-37.1</td>
<td>50.6</td>
<td>3.66</td>
<td>104.0</td>
<td>9.40</td>
</tr>
<tr>
<td>15% w/w CF HCl</td>
<td>-37.4</td>
<td>63.8</td>
<td>2.63</td>
<td>107.6</td>
<td>8.49</td>
</tr>
</tbody>
</table>

Transition temperatures were also measured following gamma irradiation, and then once
again following the 28-day degradation study. Gamma irradiation was found to slightly increase
the glass transition temperature, $T_g$, of the electrospun scaffolds by ~3°C, and caused an increase
in $T_{m1}$ for the 0% w/w CF and 7 and 15% w/w CF (AO) scaffolds (from 46.0, 51.3 and 50.6 to
60.7, 58.5, and 58.1°C respectively) (**Supplemental Fig. 4S-6**). The change in each parameter
between the starting gamma irradiation state and following the degradation study of these
samples is shown in Figure 4-7. The 28-day degradation study had a very different effect on the
microstructure for the 0% w/w CF (AO) scaffolds when compared to those scaffolds with AO or
CF incorporated. For the 0% w/w CF (AO) scaffolds, $T_g$ and the melt transition temperatures $T_{m1}$
and $T_{m2}$ have decreased. In contrast, the scaffolds with AO or CF HCl showed no change or
slight increases in $T_g$ and increases in $T_{m1}$ and $T_{m2}$.

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Figure 4-7. CF and AO affect the change in PCNU transition temperatures following hydrolytic degradation. The change in the glass transition temperature ($T_g$) as well as melt transition temperatures and their associated enthalpies ($T_m$ and $\Delta H$) for the electrospun scaffolds following degradation compared to the pre-incubated gamma irradiated scaffolds. $T_{m1} = \text{first melt transition temperature (soft segment)}$, and $T_{m2} = \text{second melt temperature (hard segment)}$.

4.3.6 Antimicrobial efficacy

The MIC of released CF against *P. gingivalis* is reported in Table 4-3. The MIC of cumulatively released CF was compared to CF HCl off-the-shelf. The MIC of CF released from the scaffolds is slightly higher than that of CF off-the-shelf. This may be due to thermal and/or photo-degradation of the CF in solution over the 28 day study (Turel and Bukovec 1996; Hubicka et al. 2013). The MIC of CF from the 7% w/w CF (AO) scaffold is slightly higher than that of the CF from the 15% w/w CF (AO) and CF HCl scaffolds. Polyurethane-related degradation products present in higher amounts in the 7% w/w CF (AO) degradation solution (Figure 4-3) may be interacting with the CF and further reducing its activity.
Table 4-3. Cumulatively released CF from electrospun fibres is active against *P. gingivalis*. MIC of cumulatively released CF from scaffolds compared to CF HCl dissolved in Milli-Q water (off-the-shelf) for *P. gingivalis* 33277.

<table>
<thead>
<tr>
<th>Degradation and Drug Release Products</th>
<th>MIC of CF (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% w/w CF</td>
<td>N/A</td>
</tr>
<tr>
<td>7% w/w CF (AO)</td>
<td>0.488 &lt; MIC &lt; 0.975</td>
</tr>
<tr>
<td>15% w/w CF (AO)</td>
<td>0.319 &lt; MIC &lt; 0.638</td>
</tr>
<tr>
<td>15% w/w CF HCl</td>
<td>0.319 &lt; MIC &lt; 0.638</td>
</tr>
<tr>
<td>CF HCl (off-the-shelf)</td>
<td>0.156 &lt; MIC &lt; 0.313</td>
</tr>
</tbody>
</table>

4.3.7 Cell compatibility with degradation products released from scaffolds

In anticipation of future applications for gingival tissue engineering, the compatibility of the antimicrobial scaffold degradation products was evaluated using human gingival fibroblasts, the cells that contribute to the maintenance and remodelling of the gingival connective tissue (Taba et al. 2005; Giannopoulou and Cimasoni 1996). Figure 4-8 reports the relative metabolic activity of HGF-1 exposed to 3 different concentrations of degradation and drug release products from day 7 of the study (24 hour exposure), as drug/polymer related products accumulated > day 7 of the study were projected to be at a higher level of accumulation than that which is clinically relevant, and may have had the potential for aging effects such as thermal or photodegradation (Turel and Bukovec 1996; Hubicka et al. 2013).
Release products from all scaffolds were found to have an effect on HGF metabolic activity. All scaffolds showed a small but significant reduction in metabolic activity at the highest concentration of release products (90%), except the 15% w/w CF (AO) scaffolds which showed a marginally statistically insignificant decrease. Since each scaffold showed a similar reduction in metabolic activity in the presence of 90% v/v degradation products, it can be deduced that the CF and AO did not have a cytotoxic effect on the HGF. Rather, hydrolytic degradation products and/or other extracts from the PCNU likely caused the change in metabolic activity. Cell numbers were assessed by also measuring DNA/ RNA content. Although metabolic activity was reduced, DNA and RNA content remained unchanged (Supplemental Fig. 4S-7), suggesting that the cell numbers were not affected by the presence of the degradation products. XPS results showed a Sn concentration associated with the polyurethane’s catalyst was on the order of 0.05±0.06 atomic%, below the limit of detection for XPS (0.1 at%) so a cytotoxic effect due to leached organo-tin catalyst DBDL used in the PCNU synthesis is not likely. Residual electrospinning solvent was thoroughly washed following scaffold fabrication; as confirmed by the absence of fluorine in the 0% scaffold controls analysed via XPS.
To further explore the hypothesis that PCNU related degradation products could be associated with the changes in HGF metabolic activity, the cytocompatibility of 0% w/w CF scaffold degradation products from day 1 and day 28 of the study were compared to the day 7 degradation products (Supplemental Fig. 4S-8). Degradation products from day 1 showed no effect on metabolic activity, while by-products at 90% v/v from day 7 and day 28 showed a significant reduction in metabolic activity compared to the growth medium control, in a dose dependent manner between days 1, 7 and 28. Again, the DNA/RNA content was not significantly affected.

### 4.4 Discussion

Electrospun PCNU nanofibre scaffolds have been previously evaluated for use in annulus fibrosis (AF) tissue engineering, in which the scaffold can promote AF cell attachment and ECM production (Yang et al. 2009), transfer topographical and tensional cues (Turner et al. 2014), and maintain scaffold shape and modulus following initial hydrolytic degradation (Yeganegi, Kandel, and Santerre 2010). In the current work, this scaffold platform was modified via the incorporation of antimicrobial agents which could be used in future application in tissue engineering of gingival tissues where free bacteria reside in the implant environment. The use of a synthetic material in the infectious oral environment has the potential to lead to biofilm infections that may delay or inhibit healing in periodontal tissue regeneration strategies despite systemic administration of antibiotics (Bottino et al. 2012). Antibiotics may be incorporated into electrospun scaffolds directly, although the almost inevitable burst release of drug leads to difficulty in optimizing dosing for longer-term applications (He, Huang, and Han 2009; Gilchrist et al. 2013a; Reise et al. 2012; Ruckh et al. 2012). The AO used in this study is hypothesized to potentially enable a more sustained release of CF from nanofibre scaffolds of PCNU fabricated via blend electrospinning, as the polymeric CF must by hydrolysed before it has any bioactivity (Santerre and Esfand 2014), and may increase favourable interactions between the drug and the polyurethane scaffold matrix to slow the diffusion of drug from the scaffolds (Wright et al. 2017). The requirement for a slow and sustained release of antibiotic at concentrations above the MIC of target pathogens was an important design consideration in the current system. This is one of many important considerations required to counter the conditions associated with the development of antibiotic resistant bacteria (Smith 2005). CF was chosen for use due to its
broad-spectrum of activity and excellent tissue penetration (Slots and Rams 1990), however the current system may be adapted to include other antibiotics.

Researchers have investigated different factors that affect the rate of release for loaded drugs such as drug type and form (Gilchrist et al. 2013a), and have explored various means of slowing release from the scaffolds, for example using emulsion or coaxial spinning to create core-shell fibres (Xu et al. 2005; Huang et al. 2006), or by incorporating drug loaded nanoparticles into the nanofibres (“nano in nano” technique) (Khodir et al. 2013). The literature in this area is overwhelmingly focused on the release kinetics of drug from the scaffolds while little attention has been given to the effect of the added drug on scaffold degradation characteristics.

The rate of scaffold biodegradation for drug delivery will ultimately affect the release of drug from scaffolds used in longer-term applications. In addition, drug-loaded materials that also act as tissue engineering scaffolds must address the important question of the effect of biodegradation products on cells related to tissue integration and tolerance. Within the broader field of polymer chemistry, small molecular weight additives of all different forms are becoming increasingly common and the field as a whole must address how these additives will affect the matrix biodegradation properties in order to successfully bring them to the clinic (Carlsson and Chmela 1990).

In the current study, fibre diameter was maintained between all scaffolds with varying drug compositions, and the study took place over a timeframe in which fibre degradation is known to occur (Yeganegi, Kandel, and Santerre 2010). Fibre diameter and alignment were maintained uniform during processing between samples by altering the concentration of PCNU in the electrospinning solutions and by changing the rotational speed of the mandrel collecting surface. The introduction of AO and CF HCl necessitated an increase in the PCNU solution concentration to adjust the apparent viscosity and generate uniform fibre diameters between all scaffolds, and the speed of the rotating mandrel was increased to adjust fibre alignment (Table 4-1). As such, since the porosity and fibre diameter of the scaffolds was kept constant, it can then be concluded that they were not significant factors contributing to the observed changes in drug release kinetics or hydrolytic degradation. It was hypothesized that the added CF HCl and AO
could affect the H-bonding character, phase mixing and microstructure of the polyurethane fibres, which in turn could influence the rate of nanofibre scaffold hydrolysis.

The AO was hydrolysed to release CF slowly over the duration of the study (Figure 4-2), so that while the scaffolds with 15% w/w CF HCl had released about 500 µM of free CF drug within the first hour of the study, the scaffolds with 15% w/w CF (AO) had only contributed about 5 µM of free CF. By incorporating the CF as part of the AO, a more sustained generation of CF is possible. In a previous study completed by the authors, this effect was revealed to be at least partly a result of incorporating CF as part of the AO which has a TEG spacer (Wright et al. 2017). The AO increases favourable PCNU/drug interactions due to the unique character of TEG which is often used as diluent agent to promote movement of polymer chains, thereby allowing for the production of fibres with more uniformly distributed drug. Additionally, the AO must be hydrolyzed before free CF is released (Wright et al. 2017). The AO continues to generate free CF for the entire duration of the study, up to day 28. Within the parameters of this in vitro system, the incremental production of CF at each time point for both the 7 and 15% w/w CF (AO) scaffolds is above the MIC of CF for characteristic periodontal pathogen P. gingivalis (determined as between 0.5 µM and 0.9 µM in the current study, similar to other studies (Eick et al. 2004)). The accumulated CF from the degradation solution had an MIC slightly higher to that of off-the-shelf CF HCl. It is thought that this could be related to processing and handling conditions (e.g. thermal and photodegradation (Turel and Bukovec 1996; Sinica et al. 2012; Hubicka et al. 2013)) over the 28 day incubation period (Table 4-3). An alternative explanation is the possible complexation with PCNU degradation products, which may have altered activity. This remains to be further investigated, but given the marked changes in the polar character and domain structure for the PCNU in the presence of CF HCl and AO (Figure 4-6 and Figure 4-7), there is strong rationale to consider this hypothesis. However, overall, the incorporation of the CF into AO form and the electrospinning process did not appear to have a substantial detrimental effect on the antibiotic bioactivity, as cumulated CF in the degradation solutions had a similar MIC to that of CF HCl released from the polymer.

The cell compatibility of the scaffolds was assessed with HGF-1. It was hypothesized that the 15% w/w CF HCl scaffolds may have potential cytotoxicity to local cells upon implantation due to the high concentration of free drug initially released. Contrary to the initial hypothesis, the
release of CF at high concentrations did not have any differential effect on HGF metabolic activity when compared to the 0% w/w CF scaffolds. Rather, all the scaffolds had a similar effect on the HGF metabolic activity (Figure 4-8). Cellularity did not appear to be affected by the hydrolysis products (no significant difference in DNA/RNA quantity, α=0.05, Supplemental Fig. 4S-7). Accumulated hydrolytic degradation by-products at day 28 were also investigated for the PCNU (Supplemental Fig. 4S-8), and a further decrease in metabolic activity was observed, without a change in DNA/RNA content. Since release products from day 1 (a time by which extractable solvent would have been removed) showed no effect, residual solvent or contaminants are not expected to be the source of the reduced HGF metabolic activity observed. Hexane diamine, a by-product of the PCNU hydrolysis, has previously been shown to be cytotoxic towards HeLa cells (Kim et al. 2002), and may have been responsible for the slight drop in cell metabolic function observed here. However, the solutions tested represent a concentration of degradation by-products that would not likely accumulate in the highly perfused gingival tissues, and the PCNU electrospun scaffolds (without CF) have previously been shown to be non-cytotoxic when used with annulus fibrosus cells in spinal disc avascular tissue engineering (Yeganegi, Kandel, and Santerre 2010).

The extent of scaffold degradation was measured using GPC, and the reduction in molecular weight was reported as a % of the starting weight-average molecular weight, $M_{w,PS}$ (recorded at day 0) in Figure 4-3. The addition of CF had a marked effect on the rate of hydrolysis of the scaffolds. Unexpectedly, incorporating CF in CF HCl form or AO form reduced the rate of hydrolysis when compared to drug-free scaffolds. While other investigators have shown that incorporating drug into electrospun nanofibres increases the rate of hydrolysis due to the formation of pores (Weldon et al. 2012) or by increasing the hydrophilicity of the fibres (Cui et al. 2006), the addition of AO and CF HCl (materials that increased the hydrophilic character of the scaffolds (Supplemental Fig. 4S-3) resulted in increased hydrolytic stability, even after all or most of the added drug had diffused from the scaffolds over 28 days of incubation. Furthermore, it was noted that the average fibre diameter for each scaffold increased, but the increase was particularly marked for the 0% w/w CF scaffolds (Figure 4-5). Studies involving the degradation of PLGA nanofibre scaffolds of varying PLA to PGA co-polymer ratios reveal that hydrolysis results in the eventual merging of fibres, which may proceed until individual fibres are no longer visible (Blackwood et al. 2008). High magnification SEM images reveal a
smooth surface morphology with no evidence of pore formation evident (**Figure 4-4**, inlay). The degradation results suggest the CF and AO have a lasting effect on the scaffold hydrolytic stability, unrelated to surface chemistry or fibre morphology.

Polyurethanes are segmented materials comprised of hard and soft micro-domains, and the phase structure and domain features have been shown to influence PCNU degradation processes (Tang, Labow, and Santerre 2001a, 2001b). Scaffold microstructure was investigated via DSC. The soft segment PCN may exist in either amorphous or crystalline form and a higher degree of crystallinity is known to increase hydrolytic stability (Zong et al. 2003), but phase mixing and the degree of H-bonding between the different segments of the PCNU are also important characteristics that will define the susceptibility of hydrolytically sensitive functional groups (Tang, Labow, and Santerre 2001a, 2001b). Hence, the H-bonding character of the electrospun scaffolds’ PCNU matrix was investigated.

In PCNUs, strong H-bonding interactions can occur within the hard segment urethane amines and carbonyls, as well as between the hard segment amines and soft segment carbonate carbonyls. The addition of the AO was found to significantly increase the ratio of H-bonded carbonate carbonyls, while CF HCl caused an even further increase in the ratio of H-bonded carbonate carbonyls and reduced the ratio of H-bonded urethane amines (**Figure 4-6A**). Transition temperatures measured via DSC revealed a marked increase in the soft segment melt temperature for the 15% w/w CF HCl scaffolds indicative of an increase in soft segment mixing (even relative to the 15% CF (AO) scaffolds which have the same amount of CF incorporated in oligomeric as opposed to CF HCl), further suggesting that the CF incorporated in these scaffolds is localized in the soft segment domain (**Table 4-2**). The AO in turn may be interacting more favourably with the hard segment, as the ratio of H-bonded to non-H-bonded urethane carbonyls significantly decreased following the release of most of the AO by day 28 (**Figure 4-6**). The melt transition temperature associated with the hard segment ($T_{m2}$) became less pronounced upon the addition of the AO (**Table 4-2**) indicative of a decrease in the size/purity of the crystalline domain within the hard segment, supporting the hypothesis that the AO is interacting with the hard segment.
Following the 28 day degradation study, the ratio of H-bonded to non-H-bonded amines decreased for the 0% w/w CF and 7% w/w CF (AO) scaffolds, while it had slightly increased for the 15% w/w CF (AO) and CF HCl scaffolds. The ratio of H-bonded to non-H-bonded urethane carbonyls became significantly lower for the 0% w/w CF scaffolds compared to the scaffolds with added drug. A decrease in H-bonding in the hard segment likely contributed to the greater extent of hydrolysis in the 0% w/w CF scaffolds when compared to the scaffolds with drug. Both the carbonyl and urethane groups are less susceptible to hydrolysis if involved in H-bonding interactions, due to a hindrance effect and the effect of the electropositive proton donor which pushes electrons away from the C=O bond and towards the hydrolytically sensitive C–O, rendering the latter more stable. It is the H-bonded urethanes, however, that were shown to increase the stability of PCNUs in the earlier stages of degradation (Tang, Labow, and Santerre 2001a), in part because of the hard segment’s ability to act as a shield to the more hydrolytically sensitive polycarbonate soft segment (Tang, Labow, and Santerre 2001b).

The transition temperatures of the scaffolds were all affected by the degradation process. The $T_g$ of the 0% w/w CF decreased, as did the soft and hard segment associated melt transition temperatures (Figure 4-7). The $T_g$ and $T_m$ of pure PCN macromonomer is -60°C and 31°C respectively, and as such a decrease in the $T_g$ or $T_{m1}$ of PCNU is indicative of an increase in the amount of micro-phase separation (Tang, Labow, and Santerre 2001a). This would accompany a relative drop in H-bonding within the soft segment. There was also an increase in the magnitude of both $\Delta H_1$ and $\Delta H_2$, further indicating an increase in the separation of the soft and hard segment phases. The crystallinity of the soft segment may be increasing as the amorphous regions are preferentially degraded (Zong et al. 2003). It has been suggested that following water uptake by PCNUs, phase separation increases due to preferential H-bonding interactions between the more polar urethane groups and water molecules (Chen and Hsu 1993; Tang, Labow, and Santerre 2001a). Interestingly, the $T_g$ of the AO-loaded scaffolds and the $T_{m1}$ and $T_{m2}$ of the AO and CF HCl loaded scaffolds increased following the 28 day degradation study, and $\Delta H_1$ and $\Delta H_2$ decreased. So, while phase separation increased in the 0% w/w CF scaffolds, there is greater phase mixing in the scaffolds with drug. This may have led to shielding of the susceptible carbonate soft segment, with the result that the drug-loaded scaffolds have greater hydrolytic stability (Figure 4-9).
For the current study, evidence suggests that the AO and CF HCl added to the polyurethane alter the hydrolysis kinetics through altering H-bonding interactions and microstructure organization within the scaffold matrix. The change in H-bonding appears to be relatively stable within the timeframe of the current experimental conditions – affecting the scaffolds even after all or almost all the AO or CF has been released (Figure 4-7). Further studies will examine the effect of other antibiotics or small molecular weight additives on the degradation rate of PCNU electrospun scaffolds in order to better elucidate the mechanism behind the effect of added AO and CF on H-bonding, microstructure and degradation rate. The effect of added drug on the degradation of PCNU in film form may also be examined to elucidate the role of electrospinning in the observed effect on hydrolysis rate.

The observed increase in the hydrolytic stability of the 15% w/w CF (AO) scaffolds may in fact be beneficial for the intended gingival tissue engineering application, as the scaffold must
retain shape and form for approximately 4-6 weeks during wound healing and tissue integration of gingival constructs. Furthermore, a sustained release of antibiotic above the MIC for the lifetime of the implant is desired for long-term prevention of a biomaterial-associated infection, and has been suggested as being a condition that would potentially contribute to reducing the chance of bacterial antibiotic resistance development (Smith 2005). As such, the 15% w/w CF (AO) scaffolds represent a scaffold platform with sought-after functionality for potential use in gingival tissue engineering.

4.5 Conclusion

The results of the current study indicate the ability to use CF in the form of the AO to generate electrospun scaffolds that can generate a sustained (>28 days) accumulation of CF. Moreover, it was found that added drug (in AO or as CF HCl) increased the hydrolytic stability of the PCNU scaffolds by altering the H-bonding and phase mixing of the PCNU matrix. These results highlight the importance of characterizing degradation properties of drug-loaded scaffolds. The combination of sustained drug release and delayed scaffold degradation presents a viable treatment modality for medical device applications requiring long term antimicrobial management such as gingival tissue engineering.

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Disclosure of Conflict of Interest

Author Dr. Paul Santerre is engaged as a senior contractual consultant for Interface Biologics Inc.
4.6 Supplementary Data

**Supplemental Fig. 1.** The AO is in the form of a trimer and consists of two CF molecules covalently linked to TEG via hydrolysable ester bonds.

**Supp. Fig. 4S-2.** Low magnification SEM images of electrospun scaffolds with (A) 15% w/w CF (AO) and (B) 15% w/w CF HCl. A clump of aggregated CF is denoted by white arrowhead.

**Supp. Fig. 4S-3.** Static water contact angle on aligned fibre scaffolds. * Represents significant difference between groups, † significant difference from all other groups (p < 0.05). Data are the mean ± SD (n=5).
Supp. Fig. 4S-4. Characteristic peaks from ATR-FTIR absorbance (abs) spectra of electrospun scaffolds.
Supp. Fig. 4S-5. DSC thermograms of as-made electrospun nanofibre scaffolds with (A) 0% w/w CF, (B) 7% w/w CF (AO), (C) 15% w/w CF (AO) and (D) 15% w/w CF HCl.

Supp. Fig. 4S-6. The change in the glass transition temperature ($T_g$) as well as melt transition temperatures and their associated enthalpies ($T_m$ and $\Delta H$) for the electrospun scaffolds following gamma irradiation. $T_{m1} = $ first melt transition temperature (soft segment), and $T_{m2} = $ second melt temperature (hard segment).
Supp. Fig. 4S-7. Relative DNA/RNA content (%) for HGF exposed to Day 28 biodegradation release products from each scaffold type. * Represents significant difference from growth medium control (p < 0.05). Data are the mean ± SD (n=9).

Supp. Fig. 4S-7. Relative metabolic activity (WST) and DNA/RNA content of HGF as a % of the growth medium (GM) control after exposure to 0% w/w CF scaffold release products from day (D) 1, 7 and 28 of the biodegradation study (diluted to 90% v/v with FBS). * Represents significant difference from growth medium control (p < 0.05). Data are the mean ± SE (n=3-9).
Chapter 5

5 Harnessing Prefusion Flow in In Vitro Co-Cultures for Engineering Functional Microvessels in Synthetic Polyurethane Random-Pore Scaffolds

This chapter reports the completion of Objective 2 of this thesis, wherein an anti-infective gingival tissue construct was generated via in vitro prevascularization of D-PHI scaffolds. The chapter investigates the use of perfusion flow culture of HGF and HUVEC co-cultures on D-PHI as a means of promoting EC organization into a network that anastomoses with the host vasculature once implanted in vivo.

Highlights:

- A perfusion bioreactor for perfusion culture of cells on D-PHI scaffolds was custom designed and built.
- Three different flow rate magnitudes were investigated. A flow rate of 0.05 mL/min resulted in a greater lumen density in the constructs relative to 0.005 and 0.5 mL/min, indicating the critical importance of flow magnitude in establishing microvessels.
- Constructs generated at 0.05 mL/min perfusion flow were implanted in a mouse subcutaneous model and a novel means of imaging the engineered vessels and assessing their functionality was established.
- Engineered microvessels were functional (i.e. perfused with host blood and non-leaky).

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Abstract

Recently reported biomaterial-based approaches toward prevascularizing tissue constructs rely on biologically or structurally complex scaffolds that are complicated to manufacture and sterilize and challenging to customize for clinical application. In the current work, a prevascularization method for soft tissue engineering that uses a non-patterned and non-biological scaffold is proposed. Human fibroblasts and HUVECs were seeded on an ionomeric polyurethane-based hydrogel and cultured for 14 days under medium perfusion. A flow rate of 0.05 mL/min resulted in a greater lumen density in the constructs relative to 0.005 and 0.5 mL/min, indicating the critical importance of flow magnitude in establishing microvessels. Constructs generated at 0.05 mL/min perfusion flow were implanted in a mouse subcutaneous model and intravital imaging was used to characterize host blood perfusion through the construct after 2 weeks. Engineered microvessels were functional (i.e. perfused with host blood and non-leaky) and neovascularization of the construct by host vessels was enhanced relative to non-prevascularized constructs. We report on the first strategy toward engineering functional microvessels in a tissue construct using non-bioactive, non-patterned synthetic polyurethane materials.
5.2 Introduction

Gingival recession is an example of a clinical soft tissue defect for which regeneration may be accomplished using tissue engineering strategies. Gingival recession affects half of all adults in the United States (Eke et al. 2012), and a tissue engineered gingival construct would mean a less painful procedure and faster recovery relative to the current gold standard treatment – connective tissue autografts (Moharamzadeh et al. 2012). In reconstructing fibrous connective tissue like the lamina propria layer of the gingiva, a tissue engineered construct should mimic the natural tissue, and must survive and integrate into local tissue once implanted in vivo. Toward this goal, it is essential to generate a functional microvasculature within the construct in vitro that will quickly anastomose with the host vasculature upon implantation and promote further vascularization via host angiogenic processes. Tissues in a full-thickness construct that are not readily vascularized in vivo will become necrotic, and integration of the construct with the surrounding host tissue will be slow or non-existent (Auger, Gibot, and Lacroix 2013). In addition, for applications such as in the oral cavity where synthetic biomaterial scaffolds may be prone to biofilm-associated infection once implanted, rapid access to the natural host defense mechanisms via perfusion of blood is critically important (Slots, MacDonald, and Nowzari 1999).

Prevascularization of tissue constructs is an active area of research within the field of biomaterials for tissue engineering, whether for soft tissue (Frueh et al. 2017), muscle tissue (J. J. Kim, Hou, and Huang 2016), or for hard tissue applications (Santos and Reis 2010). Yet elucidating a successful prevascularization strategy remains one of the field’s major challenges (Laschke and Menger 2016). Since vasculogenesis and angiogenesis processes (the de novo formation of vessels and sprouting of new vessels from existing vessels, respectively) are also studied in the field of biomaterials in applications relating to tumor models and organoid manufacturing for the study of disease states, there is a wealth of literature on different strategies for vascularizing a tissue construct in vitro.

Strategies for engineering microvessels in a biomaterial scaffold generally involve engineering a sophisticated pro-angiogenic scaffold material by incorporating geometric cues (Mirabella et al. 2017), mechanical cues (Chen et al. 2012), or pro-angiogenic biomolecules such as growth factors (Amaral et al. 2018) and extracellular matrix (ECM) proteins (Landau et al.
It is also common to employ a co-culture technique, as endothelial cells (ECs) often fail to organize or survive in a construct long-term without supporting cells such as fibroblasts, mesenchymal stem cells (MSCs) or other tissue-specific cells (Battiston et al. 2014).

Culturing the cell-seeded scaffolds under dynamic culture conditions, i.e. within a perfusion bioreactor, can activate shear-induced biochemical signalling pathways to promote EC invasion and organization (Kaunas, Kang, and Bayless 2011). Perfusion of growth medium can also guide angiogenesis processes by improving diffusion of gases and nutrients, and significantly affecting protein and macromolecule gradients within tissue matrices via convection (Shirure et al. 2017; Griffith and Swartz 2006). Finally, hypoxic culture conditions may also be employed as a means of encouraging EC self-assembly via hypoxia-induced signalling pathways, though only a small number of studies have thoroughly investigated how oxygen gradients within the tissue matrix and/or scaffold affect angiogenesis (Phillips, Birnby, and Narendran 1995; Ottino et al. 2004).

Scaffolds that incorporate mechanical cues via micropatterning or 3D bioprinting of vessel-scale channels allow for close control over the engineered vessel geometry, and engineered microvessels with organized geometry have been shown to accelerate anastomosis with host vessels upon implantation in vivo (Baranski et al. 2013). However, survival and patency of the implanted networks are not always maintained long-term (Mirabella et al. 2017; Carmeliet and Jain 2011; B. Zhang et al. 2016), and in some cases controlling geometry did not improve perfusion of the graft in vivo (Riemenschneider et al. 2016). Additionally, the controlled network geometries produced via micropatterning or 3D printing do not recapitulate the multi-directional microvessel networks present in vivo (Morgan et al. 2019), nor are the micropatterned scaffolds conducive to generating full-thickness grafts, as the vessel channels are organized in a planar manner. Hence, this biomaterial-based prevascularization strategy is limited as a translational strategy due to current issues associated with scaling and commercializing 3D bioprinters and bioreagents (Elomaa and Yang 2017).

While incorporation of biochemical cues via embedded biomolecules can aid in methods that rely on EC self-assembly, this method also has drawbacks as a fully translational strategy. Peptides, growth factors, ECM proteins, and other commonly employed pro-angiogenic
bioactive molecules are expensive, challenging to sterilize once in the scaffolds, and difficult to standardize in preparation. Biomolecules incorporated into the as-made biomaterials would limit the shelf-life of scaffolds used for tissue engineered constructs in the clinic. While the benefits of using a synthetic polymer scaffold versus a natural scaffold material are well recognized (Drury and Mooney 2003; O’Brien 2011), the majority of prevascularization strategies rely on the use of natural polymer materials as a starting point. These materials include fibrin or collagen, which are both inherently conducive to EC invasion and tube formation. If a synthetic scaffold material is used, i.e. polylactic-co-glycolic acid (PLGA), then scaffolds are typically pre-treated with fibronectin (Santos et al. 2009; Bertlein et al. 2018), or fibrin is used as a cell-carrier during seeding (Freiman et al. 2018).

Given the limitations of current strategies that rely on highly complex and/or biological scaffolds, the authors aimed to develop a method of engineering a vascularized soft tissue construct that capitalizes on the natural pro-angiogenic state produced in a co-culture of ECs and gingival fibroblasts seeded on a synthetic polymer scaffold in a perfusion bioreactor. The scaffold material chosen is the non-patterned (i.e. random pore network) and non-biological D-PHI, or degradable polar hydrophobic ionic polyurethane, developed in-house for tissue engineering applications (Sharifpoor, Labow, and Santerre 2009; X. Zhang et al. 2017). It is an ionomer with a unique heterogenous surface chemistry that has previously been reported to encourage a pro-wound healing phenotype demonstrated with macrophages (Battiston et al. 2015).

Herein, we discuss the perfusion culture conditions (i.e. flow rate) that produced a soft tissue construct with a lumen density similar to the natural lamina propria, and report on the performance of the construct using intravital imaging of in vivo host blood perfusion. The study’s findings demonstrated that that important vascular remodelling processes can be recapitulated in vitro using synthetic scaffolds, EC/fibroblast co-culture, and flow of an appropriate magnitude such that a functional microvessel network could be generated for a prevascularized tissue construct.
5.3 Materials and Methods

5.3.1 D-PHI scaffold fabrication

D-PHI scaffolds were fabricated by free radical polymerization of a divinyl oligomer cross-linker (DVO) with methacrylic acid (MAA) and methyl methacrylate (MMA). DVO was synthesized via the reaction of hydroxyethyl methacrylate (HEMA), poly (hexamethylene carbonate) diol (PCN) and lysine diisocyanate (LDI) (Sharifpoor, Labow, and Santerre 2009). DVO, MAA, and MMA were mixed at a 1:5:15 molar ratio, and porogens polyethylene glycol (PEG) and sodium bicarbonate were added at 10 and 70 wt% respectively. Initiator benzoyl peroxide (BPO) was added at 0.0032 mol/mol vinyl group. The thoroughly combined mixture was packed into 6 mm diameter, 2 mm thick discs in Teflon molds and cured in a 110°C oven for 24 h. Cured scaffolds were flushed with 15 L of ddH2O for ~48 h in order to completely remove the porogens (~80% resultant porosity (Sharifpoor, Labow, and Santerre 2009)). Scaffolds were then dried and sterilized via gamma irradiation (25 kGy, GammaCell 220, Cobalt (Co60) source, SOCAAR, University of Toronto, Toronto, Canada).

5.3.2 Perfusion co-culture of gingival fibroblasts and HUVECs

Human gingival fibroblasts (HGF-1, ATCC CRL-2014) were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% FBS and 1% penicillin/streptomycin, in T75 TCPS flasks to passage 3. HUVECs (Lonza, CC-2519) were cultured in Lonza endothelial cell growth medium-2 (EGM-2) medium also to passage 3. Co-suspensions of HGF and HUVECs were made at a concentration of 33x10^6 cells/mL (2:1 ratio of HGF:HUVECs (Cheung et al. 2015)). The suspension was seeded onto D-PHI scaffolds pre-wet in 50/50 DMEM/EGM-2 (24 h at 37°C). Each scaffold was seeded with 300,000 cells (200,000 HGF and 100,000 HUVECs). Cell-seeded scaffolds were then cultured statically in 96-well plates for 24 h at 37°C (5% CO2) before being placed in the perfusion bioreactors.

The custom-designed bioreactor consisted of a medium reservoir and a scaffold insert that screws into place in the reservoir (Figure 5-1b). The scaffold insert was designed with a press-fit channel for a single scaffold to sit vertically such that bubble-free medium flows through the entire thickness of the scaffold disc. Cell-seeded scaffolds were placed in the
bioreactors and connected via inlet/outlet tubing to a peristaltic pump, and medium reservoirs were filled with 50/50 DMEM/EGM-2 (8mL per scaffold). The entire system was placed inside a sterile tissue culture incubator for 14 days of perfusion culture at either 0.005, 0.05, or 0.5 mL/min flow rate. The medium was refreshed every 4-5 days with the exception of the first 48h of culture, after which the medium was collected to quantify the number of cells sheared off of the scaffold into suspension during that time. Cell pellets from the medium collected at 48h were quantified for cell number using a CyQUANT assay (Thermo Fisher Scientific).

5.3.3 Determination of constructs’ DNA content and cell metabolic activity

After the 14-day culture period, constructs were removed from the bioreactors and placed in 96-well plates. The metabolic activity was quantified using a water-soluble tetrazolium-1 (WST-1) assay (Roche Diagnostics). Scaffolds were rinsed with 200 µL phosphate buffered saline (PBS) and WST-1 reagent and DMEM/EGM-2 were added to each well at a ratio of 1:10 WST-1:growth medium. The cells were then incubated for 1 h at 37°C with 5% CO₂ and the absorbance was read at 450 nm using a reference wavelength of 650 nm. The metabolic activity of the cells in the construct was reported as the relative WST-1 absorbance compared to the day 0 controls, which were scaffolds seeded at the same time as the test constructs but assayed after 24 h of static culture only.

Following the WST-1 assay, scaffolds were cut up into small pieces using a 10-guage needle and mixed with lysis buffer in a DNAase-free 1.5 mL Eppendorf tube. Samples were incubated at 65°C for 60 minutes, mixing once half-way through. Samples were loaded onto a black 96-well plate. The DNA content of the samples was quantified by binding with Hoechst 33258 dye, calibrated against calf thymus DNA standards of known concentrations (previously described (Cheung et al. 2015)).

5.3.4 Construct characterization via immunofluorescence staining

Other constructs collected after the 14-day culture period were imaged using live confocal imaging (LSM710, Zeiss). Constructs were incubated in a solution of Alexa-Fluor 594 conjugated anti-human CD31 (mouse anti-hCD31, BioLegend) in DMEM, preceded by blocking
with a solution of 2% bovine serum albumin (BSA, Sigma) in PBS and 50/50 DMEM/EGM-2. Constructs were washed 3x in PBS before imaging using a 561 nm excitation wavelength.

Some constructs were collected and then rinsed with pH 7.4 PBS twice before being fixed in 4% paraformaldehyde (prepared with pH 7.4 PBS) for 20 min. Samples were processed and embedded in paraffin before sectioning into 20µm sections for immunofluorescence staining. Scaffold sections (3 sections/sample) were immunostained according to previously established protocols (Cheung et al. 2015) for von Willebrand factor (rabbit anti-vWF, abcam; diluted 1:100), fibroblast surface protein (mouse anti-FSP, Abcam; diluted 1:300), α-smooth muscle actin (mouse anti-α-SMA, Abcam; diluted 1:25), type I collagen (rabbit anti-Col I, Abcam; diluted 1:100), and/or caspase-3 (rabbit anti-caspase-3, Abcam; diluted 1:300). Cell nuclei and the D-PHI scaffolds were stained with Hoechst 33342 (1:1000). The scaffold sections were mounted with PermaFluor (Thermo Fisher Scientific) and imaged with a fluorescence microscope (LSM700, Zeiss). Four images were captured per section, with a total of 12 images per sample. Each image was processed using ImageJ, and the integrated density and/or area fraction of signal associated with each protein marker were reported and normalized to day 0 samples. Cell-seeded scaffold sections treated with only secondary antibodies served as negative controls.

5.3.5 Assessment of constructs in vivo

In a separate experiment, constructs perfused at 0.05 mL/min for the 14-day culture period were implanted subcutaneously in the dorsum of athymic female mice (NCRNU-F, 8-10 weeks, Taconic) anesthetized using ketamine and xylazine at 80 and 13 mg/kg, respectively. Constructs cultured at a flow rate of 0.05 mL/min were designated as prevascularized constructs (PV constructs) and were implanted adjacent to day 0 controls, which were non-vascularized cell-seeded scaffolds cultured in a static manner for 24 h (NV constructs). After 2 weeks in vivo, the mice were similarly placed under anesthesia. The NV construct was removed in each mouse and fixed in 10% formalin while a dorsal titanium window was simultaneously placed to expose the PV construct for live confocal fluorescence imaging, similar to protocols used to expose and image tumors in live mice (Maeda and DaCosta 2014).
After the mice had sufficiently recovered, they were transferred to the imaging facility for live confocal imaging (Zeiss LSM710 Two-Photon/Confocal, AOMF). Under anesthesia (ketamine and xylazine at 40 and 6.5 mg/Kg respectively for maintenance), mice were placed in a custom mounting device and Alexa-Fluor 594 conjugated anti-human CD31 (AF594 anti-hCD31) was administered topically within the window. Blocking and washing steps were carried out using 10% FBS in growth medium and PBS respectively. Immediately before imaging, fluorescent blood pooling agent FITC-labeled dextran (2x10^6 g/mol, 0.1 mg/mouse) was injected via tail vein. Imaging took place using a heated stage with excitation wavelengths of 561 nm and 488 nm for AF594 and FITC, respectively.

Five regions of interest (ROI) were captured per mouse in a z-stack with image size of 607µm x 607µm, and a depth of ~300µm. A maximum intensity projection of each ROI was generated using ImageJ, and the projection was then sub-divided into 36 sub-regions. The frequency of the events of a AF594 hCD31 positive signal (red), a FITC-dextran positive signal (green), or a co-stain (yellow) were measured by counting the occurrence of one or more of these events in each sub-region in each ROI and then normalized to the total number of events. Immediately following imaging, each mouse was sacrificed for ex vivo tissue histological processing and staining. Lumens were quantified in 4 sections of each sample using the standard hematoxylin & eosin (H&E) stain.

5.3.6 Statistical analysis

All in vitro characterization of the tissue constructs was performed in triplicate with n=3 samples per condition per repeat (n=9 total samples per condition). The in vivo assessment was performed 8 times with 8 different mice. Each mouse was implanted with both the test (PV) and control (NV) constructs. Statistical analyses were performed using SPSS software (IBM, New York, version 22). Student’s t-test was used to determine the statistical significance of any differences between the mean values of two groups. In the case of more than two groups a one-way ANOVA was used. When statistical significance was found, post hoc analysis was used to determine which of the groups contributed to the statistical significance. Levine’s test of homogeneity of the variances was conducted. Tukey’s range test was used in the case of equal variances. In the event that the variances were unequal, the unequal variance version of ANOVA (Welch) was
used and Dunnett’s T3 was applied for post hoc analysis. In all analyses, the significance threshold was set to \( \alpha = 0.05 \).

5.4 Results

5.4.1 Optimal perfusion flow rate enhances microvessel organization in vitro

Prevascularized tissue engineered constructs were fabricated via perfusion co-culture of HGF and HUVECs (Figure 5-1a and b). Three different flow rate magnitudes were investigated: 0.005, 0.05, and 0.5 mL/min. These flow rates correspond to fluid velocities of \( \sim 7, 70, 700 \, \mu m/s \) respectively, which represent physiological interstitial flow, an intermediate flow rate, and a rate that is expected to produce shears experienced by ECs in capillaries (sprouting shears) (Chary and Jain 1989; Kaunas, Kang, and Bayless 2011). Shears were estimated based on a previously reported model (Radisic et al. 2008) (see Supplementary Data). Constructs were collected from the bioreactors after 14 days of perfusion culture, and these constructs underwent in vitro characterization, including immunofluorescence staining of the HUVECs and HGF to visualize the engineered microvascular network (Figure 5-1c). The average integrated density of the signals associated with HUVECs and FSP, normalized to day 0, were quantified and are reported in Figure 5-1d. There was a significantly greater average integrated density of the signal associated with FSP at the high flow rate, indicating that HGF proliferated at a greater extent at 0.5 mL/min than at 0.005 or 0.05 mL/min. The intermediate flow rate had a significantly greater average integrated density of vWF signal, which indicates that this flow rate was most amenable to HUVEC proliferation.

Furthermore, the constructs generated at the intermediate flow rate of 0.05 mL/min were found to contain a significantly greater average lumen density when compared to those generated at the low and high flow rates (Figure 5-1e and f). The lumen density reported for this flow rate was \( \sim 5 \, \text{lumens/mm}^2 \), which is close to the lumen density found in the natural gingiva (\( \sim 10 \, \text{lumens/mm}^2 \)) (Bullon et al. 2004). The flow rate magnitude did not have a significant effect on the average perimeter of the vessels generated. The average lumen perimeter (\( \sim 150-250 \, \mu m \)) and diameter (\( \sim 50-80 \, \mu m \)) measured in the constructs fall between the dimensions reported for a typical arteriole.
Figure 5-1. EC organization at varying magnitudes of perfusion flow. (a) Micro-computed tomography (CT) 3D scan of the D-PHI scaffold. (b) Custom perfusion bioreactor schematic. (c) TOP: Immunofluorescent-stained sections of tissue constructs at day 14. Red = von Willebrand Factor (vWF), Green = fibroblast surface protein (FSP), Blue = Hoechst stain (nuclei & scaffold). A typical lumen in each image is indicated with an arrow head. BOTTOM: 3D reconstructions of tissue constructs at day 14 imaged using live confocal microscopy and stained with AF594-anti-humanCD31. (d) Quantification of FSP and vWF expression (average integrated density) for each flow rate at day 14. (e) Quantification of average lumen density for each flow rate at day 14. (f) Quantification of average lumen perimeter for each flow rate at day 14. Data are mean ± SD (n=9 scaffolds), *p<0.05.

The construct sections were also stained using immunofluorescence techniques for type I collagen, which is the most abundant ECM protein in the natural gingiva, as well as the contractile protein and myofibroblast marker α-SMA. ColI was found to be deposited within the
pores of the D-PHI scaffolds, and the lumens of the engineered microvessel network within the CollI matrix in pores were clearly evident (Figure 5-2a). As seen in Figure 5-2b, there was significantly less CollI and significantly more α-SMA signal at the high flow rate (0.5 mL/min) as compared to the low and intermediate flow rates. There are no significant differences between the signal associated with CollI or α-SMA positive staining between the low and intermediate flow rate. In some ROIs for samples generated at the intermediate flow rate (0.05 mL/min), there was colocalization of α-SMA and lumen interiors. This could be indicative of the presence of α-SMA-producing fibroblasts that are taking on a pericyte-like role and supporting maturing microvessels (Zohar et al. 2017).
Figure 5-2. Extracellular matrix and cellularity at varying magnitudes of perfusion flow.
(a) Immunofluorescent-stained sections of tissue constructs at day 14. Red = von type I collagen (ColI), Green = α-smooth muscle actin (α-SMA), Blue = Hoechst stain (nuclei & scaffold). White arrowheads indicate ColI and α-SMA co-signal. (b) Quantification of ColI and α-SMA expression (average integrated density) for each flow rate at day 14. (c) Cell metabolic activity (measured as WST-1 absorbance) and DNA content (measured as ng of DNA) at day 14 relative to day 0. (d) Quantification of caspase-3 expression (average integrated density) for each flow rate at day 14. (e) Number of cells sheared from the scaffold under perfusion at each flow rate after 3 days. Data are mean ± SD (n=9).
*Significantly different from all other groups, † Significant difference between two groups, p<0.05.

The metabolic activity and DNA content associated with constructs after 14 days of in vitro perfusion culture were measured. Cell metabolic activity at day 14 was similar in constructs generated at each flow rate, while the DNA content showed a significantly greater increase at the intermediate flow rate compared to the low and high flow rates (Figure 5-2c). Immunofluorescence staining of sample sections was used to measure the relative proportion of cells undergoing apoptosis by staining for apoptotic marker caspase-3 and quantifying the
average integrated density (Figure 5-2d). There was significantly greater amount of positive staining for caspase-3 at the lowest flow rate, which indicates the greatest proportion of apoptotic cells at this flow rate. Constructs generated at this flow rate had ~4x apoptotic marker than those at day 0, while constructs generated at the intermediate flow rate (0.05 mL/min) had much less apoptotic marker than the samples at day 0.

The cell-seeded scaffolds submitted to the highest flow rate had a significant portion of cells sheared off the scaffold within the first 48 hours of culture (Figure 5-2e). Several thousands of the 300,000 cells that were seeded on the scaffold were sloughed off into the medium at this flow rate, as determined by counting the number of cells via a CyQUANT assay in the suspension of cells collected from the bioreactor medium. Very few cells were found in the medium of cell-seeded scaffolds perfused at the low and intermediate flow rates.

5.4.2 Engineered microvasculature promotes anastomosis and neovascularization in vivo

Soft tissue constructs generated at the intermediate flow rate (0.05 mL/min) were implanted subcutaneously in athymic mice to assess the functionality of the engineered microvessels using live confocal imaging. As seen in Figure 5-3a, perfused, non-leaky engineered vessels within the constructs were co-stained with FITC and AF594 signal from the dextran and hCD31 antibody respectively (FITC+, AF594+). In some regions, perfused host vessels were evident in the construct (FITC+, AF594-). In yet other regions, engineered vessels in the constructs were non-perfused (FITC-, AF594+). The relative frequencies of each of these “events” in the constructs are reported in Figure 5-3b. The frequency of unorganized HUVECs seen in the constructs in vivo is also reported. Each of these events occurred at a similar frequency in the constructs examined.
Figure 5-3. Characterization of vessels in constructs after 14 days in vivo. (a) 3D image of microvasculature in the engineered constructs captured via intravital confocal fluorescence microscopy z-stacks. Engineered vessels were stained red via the topical administration of AF594 conjugated anti-human CD31. FITC-dextran was injected intravenously prior to imaging, therefore perfused vessels are FITC+ (green). Vessels that are hCD31+ and FITC+ are co-stained (yellow). (b) Relative frequency of perfused engineered vessels (hCD31+, FITC+), nonperfused engineered vessels (hCD31+, FITC-), perfused mouse vessels (hCD31-, FITC+), and unorganized HUVECs (hCD31+) in sub-sections of n=5 ROIs per mouse. Data are mean ± SD (n=8).

To evaluate the utility of a PV construct vs. a NV construct in establishing highly perfused grafts in vivo, the number of lumens in histological sections of PV constructs versus the NV controls implanted adjacent to the PV constructs were counted. Typical ROI in histological sections stained with H&E are shown in Figure 5-4a and the average lumen density for NV versus PV constructs is reported in in Figure 5-4b. There was a significantly greater lumen density in the constructs after 2 weeks in vivo when the constructs were prevascularized. The PV constructs had a lumen density of ~8 lumens/mm² which is very close to that of highly perfused natural tissues such as the natural gingiva (~10 lumens/mm²) (Bullon et al. 2004). The PV constructs are also more cell- and tissue-dense relative to the NV controls.
5.5 Discussion

Tissue engineering has great potential for treating soft tissue loss or damage. Regenerative medicine technologies currently in the clinic for the treatment of gingival recession, periodontal disease, and trauma to the oral mucosa are limited in their ability to increase gingival thickness
and attachment in part because they are not vascularized and thus cannot functionally maintain a full-thickness graft that is dense with cells and tissue (Kress et al. 2018). Furthermore, a synthetic scaffold that is implanted in the infectious oral environment may be prone to colonization by oral bacteria, and prevascularizing the construct may aid in infection-control. However, engineering functional microvasculature within a tissue engineered construct remains a major translational hurdle faced by researchers in the field (Bezenah, Kong, and Putnam 2018; Pellegata, Tedeschi, and De Coppi 2018).

Biomaterial-based strategies toward generating prevascularized tissue engineered constructs typically involve incorporating pro-angiogenic biomolecules (Peters, Polverini, and Mooney 2002; Ehrbar et al. 2004) or the use of microfabrication techniques to guide EC organization via mechanical or geometric cues (Borenstein et al. 2002; Y. C. Chen et al. 2012). Scaffolds with biomolecules are expensive and difficult to standardize or customize to meet different clinical needs, while micropatterned scaffolds have shown mixed success in the literature and do not adequately replicate the arbitrary and multi-dimensional network of native microvasculature (Morgan et al. 2019). Synthetic scaffolds are more adaptable and practical for translation to the clinic; however, when synthetic scaffold materials (e.g. PLGA) are used, it is common to still pre-treat the scaffold with fibronectin (Santos et al. 2009; Bertlein et al. 2018), seed the cells within a fibrin matrix (Mirabella et al. 2017), or even functionalize the scaffold with relevant biomolecules (Landau et al. 2017).

In the current study, the synthetic polymer D-PHI (Sharifpoor et al. (Sharifpoor, Labow, and Santerre 2009)) was used to generate gingival constructs with functional microvasculature. A flow rate magnitude that optimally generates a tissue-dense and vessel-rich construct was identified, and it was demonstrated that engineered vessels in the PV construct anastomosed with the host vessel system and remained function (perfused and non-leaky) by 14 days post-implantation. The PV constructs also resulted in improved tissue integration and perfusion of the constructs relative to the NV control that was not prevascularized. To our knowledge, this is the first reported biomaterial-based strategy for prevascularization that does not rely on the use of pro-angiogenic bioactive molecules or ECM proteins within the as-made scaffold, a feature that makes it readily scalable and clinically translatable relative to other commonly proposed biomaterial-based tissue engineering strategies.
The use of D-PHI as a tissue engineering scaffold has previously been reported for both gingival tissue engineering applications (Cheung, McCulloch, & Santerre, 2014; J. W. C. Cheung et al., 2015) and vascular graft engineering (Battiston et al. 2015; Sharifpoor et al. 2010). It is an elastomeric polyurethane hydrogel that undergoes surface-mediated hydrolysis with a linear degradation profile and pH-neutral degradation products (McBane, Sharifpoor, et al. 2011). D-PHI scaffolds have a random interconnected micro- (1-5µm) and macro- (30-250µm) porous network and a heterogenous surface chemistry that includes polar, hydrophobic and ionic modalities. This specific chemistry has been shown to attenuate pro-inflammatory monocyte responses through the controlled absorption of the major pro-inflammatory serum protein immunoglobulin G (IgG) (K. G. Battiston et al. 2015). The controlled degradation of D-PHI into non-acidic degradation products and it’s proven immunomodulatory surface character make it an attractive alternative to PLGA (McBane, Sharifpoor, et al. 2011).

It was hypothesized that ECs and fibroblasts co-cultured on D-PHI scaffolds under perfusion flow would exhibit a pro-wound healing, pro-angiogenic phenotype in part due to the ability of D-PHI to mimic bulk mechanical properties of natural soft tissue, modulate protein binding, and enhance ECM production by cells, as previously seen in co-cultures of monocytes and tissue specific cells on D-PHI (Battiston et al. 2015). ECs are known to be mediators of tissue response during wound healing and regeneration, and D-PHI may act as an appropriate environment to promote paracrine, or “angiocrine”, signalling that will favour tissue regeneration and vasculogenesis over fibrosis (Rafii 2018; Pellegata, Tedeschi, and De Coppi 2018). Herein we characterized the extent of EC organization in the constructs after 14 days of in vitro perfusion culture under three different magnitudes of medium perfusion flow: 0.005, 0.05 and 0.5 mL/min. It was hypothesized that high flow rates would activate shear-mediated cell signalling pathways that would promote faster and/or more extensive and/or more aligned vessel organization (Kang, Bayless, and Kaunas 2008; Cheung et al. 2015), while lower flow rates would neither provide necessary shear-mediated cues nor provide adequate diffusion of gases, nutrients, and waste through the constructs (Li and Cui 2014).

The study’s findings demonstrated that the constructs generated at the intermediate flow rate of 0.05 mL/min contained a significantly greater average lumen density (Figure 5-1e), as well as higher DNA content (Figure 5-2c) relative to the low (0.005 mL/min) and high flow
rates (0.5 mL/min). Since there was a higher fluorescence intensity associated with the EC marker vWF at the intermediate flow rate (Figure 5-1d), the greater cellularity associated with the intermediate flow rate may reflect either or both a greater EC retention and/or greater EC proliferation. While we initially expected high shears to be more beneficial for the promotion of angiogenic sprouting, our findings align with a number of other studies that have shown that low levels of flow closer to interstitial flow are more beneficial for promoting angiogenic sprouting or vasculogenesis (Ghaffari, Leask, and Jones 2017; Helm et al. 2005; Bachmann et al. 2018; Kalyanaraman, Supp, and Boyce 2008; S. Kim et al. 2016; Morin, Dries-Devlin, and Tranquillo 2013; Moya et al. 2013; Song and Munn 2011). And while we hypothesized that cellularity would increase with increased flow rate due to shear-induced proliferative states exhibited by ECs (Ando, Nomura, and Kamiya 1987; dela Paz et al. 2012) and fibroblasts (Danciu et al. 2004; Garanich et al. 2007) in 2D culture, as well as increased diffusion of gases and nutrients at higher flows, other studies have similarly found that intermediate flow rates produce the greatest cellularity in perfused 3D constructs (Korin et al. 2007; Cartmell et al. 2003; Koch et al. 2010). We found that a significantly greater number of cells sheared off the scaffolds in the first 3 days of culture at the high flow rate (0.5 mL/min) (Figure 5-2e). We hypothesize that most of the cells sheared were ECs. HUVECs have been shown to be more sensitive to being sheared off of biomaterial scaffolds at early time points relative to fibroblasts (Feugier et al. 2005). Furthermore, high and direct flows have been shown to cause elution of pro-angiogenic growth factors and other signalling molecules from a 3D scaffold seeded with adipose stem cells (ASCs) and HUVECs, limiting angiogenesis (Bachmann et al. 2018).

Perfusion culture has been shown to increase fibroblast proliferation (Ng, Hinz, and Swartz 2005) and ECM production (Cheung, McCulloch, and Santerre 2014; Mathes et al. 2010), and increase myofibroblastic character, identified in part by an increase in the expression of the contractile protein α-smooth muscle actin (α-SMA) (Ng, Hinz, and Swartz 2005; Hinz 2007). In the current system, we saw both a significantly greater increase in FSP intensity, the signal associated with HGF, relative to day 0 for the constructs generated at the highest flow rate, as well as a greater increase in signal associated with α-SMA with increasing flow rate (Figure 5-1d and Figure 5-2b, respectively). The presence of myofibroblasts may be beneficial due to their increased production of pro-angiogenic growth factors transforming growth factor- β (TGF-

The lowest flow rate (0.005 mL/min) provided adequate conditions for EC organization within the scaffold, despite very low levels of shear stress estimated to be experienced by cells (Supplementary Data Chapter 5.7.1). A significantly greater amount of the apoptotic marker caspase-3 was expressed in cells generated at the low flow rate, which we expect to be the result of hypoxia-induced cell death (Figure 5-2d). Hypoxic conditions can be conducive to EC organization (Lamalice, Le Boeuf, and Huot 2007; Davies et al. 2011), and hypoxia-induced signalling pathways have previously been cited as governing mechanisms behind angiogenic sprouting in 3D scaffolds under perfusion flow (Malda, Klein, and Upton 2007; Rouwkema and Khademhosseini 2016; Landau, Guo, and Levenberg 2018).

The pro-angiogenic effect of biomolecules embedded in the ECM or in a bioactive scaffold depends on the dynamic manner that they are released from the matrix and their specific concentration gradient relative to the cell. In an in vitro construct under perfusion, medium flow provides a mechanical force that influences the distribution of these effector molecules. Helm et al. demonstrated a synergistic effect of perfusion flow and matrix-bound vascular endothelial growth factor (VEGF) for improving EC microvessel organization caused by the dynamic release of VEGF via the action of MMPs, and only generated due to the convective forces of low levels of interstitial flow (Helm et al. 2005). The important coupling of flow and biochemical signals (soluble and matrix-bound) has more recently been demonstrated by Shirure et al. (Shirure et al. 2017) and Ghaffari et al. (Ghaffari, Leask, and Jones 2017). These studies allow us to conclude that interstitial flow controls angiogenesis both through the direct action of mechanical force (on the matrix and on cells) and through indirect effects on morphogen gradients via convective forces. Although less studied, hypoxia and flow have also been shown to demonstrate a synergistic effect, in one case increasing the cellularity and ECM protein production of chondrocytes on polycaprolactone scaffolds (Dahlin et al. 2013). Together these studies suggest that the effects of perfusion, hypoxia, and biomolecule distribution on the tissue regenerative character of an in vitro tissue cannot be considered alone but are in fact effects with considerable interaction.
It is expected that, in the system reported here, complex interplay between chemical and mechanical signalling produced in the constructs at the intermediate flow rate acted in symphony to generate a significantly greater lumen density. In particular, the D-PHI platform acted as a suitable scaffold for which the co-culture could deposit dynamic ECM that responded to the action of the intermediate perfusion flow in terms of: (1) mechanotransduction of shear stresses that increased production of pro-angiogenic growth factors and ECM by fibroblasts/myofibroblasts (Cheung, McCulloch, and Santerre 2014); and (2) convective forces that produced controlled 3D gradients of pro-angiogenic signalling molecules (Helm et al. 2005; Ghaffari, Leask, and Jones 2017). Hypoxia-induced angiogenesis is hypothesized to have occurred at the low flow rate, though minimal contribution of mechanical force from perfusion flow is expected at this low flow rate. And while ECs at the highest flow rate were initially expected to have undergone shear-induced activation of pro-angiogenic signalling pathways, the latter shear was likely too high at too early a time point in the culture period as the ECs were sheared off the scaffold or underwent shear-mediated apoptosis. At the intermediate flow rate, the effect of oxygen gradients, cell-secreted signalling molecules, and matrix dynamics came together to produce an interconnected microvessel network that was proven to be functional in vivo.

Prevascularized constructs generated at the intermediate flow rate were implanted in a mouse subcutaneous model to assess the functionality of the engineered microvessel network. While our data demonstrated that not all the engineered vessels were perfused, we can conclude that it was just as likely that engineered vessels anastomosed and remained patent. We anticipate that a greater initial vessel density in the prevascularized constructs may have allowed for more efficient anastomosis with host vasculature. Future studies may entail increasing the vessel density via optimization of flow rate programs in the bioreactor. It is also possible that some of the nonperfused engineered vessels seen in vivo may have had an upstream vessel severed during the window surgery and so appear nonperfused at the time of imaging, or that there is a perfused but leaky engineered vessel upstream of those in the ROI. Unorganized HUVECs may be indicative of engineered vessels that did not anastomose with the host vasculature and have since regressed due to a lack of mechanical and/or chemical signalling, as during general remodelling processes (Meeson et al. 1999).
The presence of perfused host vessels (FITC+, AF594-) proves that the constructs are conducive to neovascularization. The lumen density in PV constructs went from ~5 lumens/mm$^2$ at the time of implantation to ~8 lumens/mm$^2$ after 14 days in vivo. This was a significantly greater neovascularization than that which occurred in NV controls (Figure 5-4b). The PV constructs are expected to be substantially more pro-angiogenic as they have greater cellularity and tissue density relative to the NV control (~1 lumen/mm$^2$). Engineered vessels in prevascularized engineered constructs have previously been shown to be rapidly replaced by host vessels, with conduciveness to neovascularization being cited as advantageous so as to allow for the host to determine blood supply based on tissue need (Koffler et al. 2011; Perry et al. 2018).

A limitation of the current study is that the controlling mechanism(s) by which the intermediate flow rate generate significantly greater network development is difficult to elucidate due to the complex and multifaceted effect of perfusion on tissue development, as discussed above. Computational modeling of the system may be the ideal method for determining more accurate levels of shear stress experienced by cells in the constructs during in vitro culture, as well as for modeling oxygen and growth factor gradients in the construct under each magnitude of perfusion flow; however, modeling of such a complex system in 4 dimensions to include time-dependent tissue production is onerous (Nava, Raimondi, and Pietrabissa 2013).

We demonstrated that a purely synthetic scaffold within which seeded cells undergo pro-angiogenic signalling pathways directed by ECM dynamics was shown to be directly affected by the flow conditions. Conditions at an intermediate flow rate generated a prevascularized scaffold that successfully anastomosed with host vessels in a mouse subcutaneous model and produced greater neovascularization than a non-vascularized cell-seeded control. The significance lies in the proposal and validation of a method for prevascularizing a soft tissue construct generated from a unique polyurethane chemistry that does not rely on the incorporation of exogenous biomolecules within the scaffold itself and is therefore highly suitable for clinical translation.

Future work will investigate the contribution of shear stress-induced signalling in the fibroblasts and ECs at each flow condition vs. the effects of hypoxia by quantifying characteristic proteins such as prolyl hydroxylase domain 2 (PHD2) to flag hypoxia/normoxia conditions (Landau, Guo, and Levenberg 2018), and focal adhesion activation and/or nitric
oxide-associated proteins like endothelial nitric oxide synthase (eNOS) to test for shear-induced activation of fibroblast and EC signalling pathways, respectively (Cheung, McCulloch, and Santerre 2014; Rotenberg et al. 2012). The majority of mechanistic studies investigating the vascularization of co-cultures under flow focus on EC mechanisms rather than that of supporting cells such as fibroblasts, so a mechanistic study of the two cells under co-culture flow could be enlightening.

5.6 Conclusion

A biomaterial-based approach toward prevascularizing tissue engineered soft tissue was proposed and validated. D-PHI scaffolds acted as a favourable chemical and mechanical environment for the proliferation of HUVECs and fibroblasts and the generation of ECM within the scaffold pores. An intermediate flow rate of 0.05 mL/min between physiological interstitial flows and high blood flow rate experienced in sprouting capillaries was most conducive to vessel formation. This is one of the only biomaterial-based approaches reported in the literature that does not rely on the use of bioactive macromolecules and/or an organized pre-fabricated vessel channel, and hence represents a method by which an engineered tissue that accurately resembles important elements of native tissue can be fabricated using materials and methods suitable for clinical translation.

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5.7 Supplementary Data

5.7.1 Estimation of shear stress in the scaffolds

The wall shear stress in the pores of the scaffold was estimated based on the Hagen-Poiseuille law for laminar flow through a conduit, wherein the pores are modeled as a series of conduits following a tortuous pathway. For this relation, we assume that fluid flow through the conduit is laminar (Reynolds number < 1) and that the conduit has a non-changing cross-sectional diameter that is significantly smaller than the conduit’s length. We also assume the fluid is incompressible and Newtonian, and that there is no acceleration of flow. The following equation can be used to estimate shear stress (Radisic et al. 2008):

\[ \tau_w = \eta \frac{4U}{R} \]  \hspace{1cm} (2)

where \( \eta \) is the fluid viscosity, \( R \) is the pore radius, and \( U \) is the average fluid velocity. Fluid velocity through each conduit can be estimated according to the following equation (Radisic et al. 2008):

\[ U = \frac{2HQ}{\varepsilon V} \] \hspace{1cm} (3)

where \( H \) is the scaffold thickness and \( 2H \) is the tortuous length of each pore, \( Q \) is the volumetric flow rate of medium through the scaffold, \( \varepsilon \) is the void fraction (i.e. porosity), and \( V \) is the scaffold volume.

5.7.2 Estimation of oxygen consumption in the constructs

The oxygen consumption in the constructs under perfusion flow culture can be determined via an oxygen balance as such (Radisic et al. 2008):

\[ F(C_{in} - C_{out}) = R_{O_2}N \] \hspace{1cm} (4)

where \( F \) is the flow rate of the growth medium, \( C_{in} \) and \( C_{out} \) are the dissolved oxygen concentrations in the medium at the inlet and outlet of the scaffold respectively, \( R_{O_2} \) is the rate of oxygen consumption for the given cell type, and \( N \) is the cell number.
5.7.3 EC organization at 0.05 mL/min

Figure 5-5. Split image of lumen density at high magnification with merge included. Magenta = vWF, green = FSP, cyan = Hoescht (nuclei and D-PHI).
Chapter 6

6 Conclusion

6.1 Summary and scientific contribution

The main objective of this thesis was to evaluate methods of generating gingival tissue engineering constructs. Two systems were generated based on two different polyurethane scaffold platforms: (1) electrospun PCNU blended with CF-based AO, and (2) a prevascularized random-pore hydrogel synthesized with a polyurethane cross-linker. In the first system, gingival fibroblasts were seeded in a monolayer on the scaffold, and in the second system, ECs were co-cultured with HGF using a perfusion bioreactor such that ECs organized into a microvessel network within the tissue-dense scaffold. These constructs were conceived with the important design consideration that a synthetic material implanted into the oral cavity is particularly susceptible to a biofilm-associated infection. In infection-prone clinical applications, an antibiotic may be incorporated into the scaffold material to prevent the infection of the wound and the scaffold material (Shi et al. 2011; Spicer et al. 2012). Prevascularizing the construct may be used as an alternative strategy in scaffold-based tissue engineering approaches such that the construct is readily perfused upon implantation and the host defenses can act to prevent bacterial colonization of the implant. This strategy is also employed in biomaterials research toward tissue engineering in infection-prone applications such as bone tissue engineering research (Auger, Gibot, and Lacroix 2013).

In Chapter 3 of this thesis, a strategy was proposed and validated wherein the broad-spectrum antibiotic ciprofloxacin (CF) was incorporated into a polyurethane scaffold via an antimicrobial oligomer (AO). The AO contains a linking triethylene glycol (TEG) between two readily hydrolysed CF molecules. It was hypothesized that incorporating antibiotic via blend electrospinning of AO with PCNU will promote a uniform distribution of drug throughout the scaffolds such that as the scaffold matrix degrades by hydrolysis, there will be a sustained release of antibiotic with antibacterial activity against characteristic microorganism *P gingivalis* without compromising HGF cell compatibility. As reported in Chapter 3 and the corresponding paper
(Wright et al. 2017), incorporating the CF as part of the AO improved drug distribution in the nanofibre scaffold compared to CF alone. AO was released from the scaffolds in a diffusion-controlled manner, and the AO was hydrolysed to release CF slowly and in a linear manner over the duration of the study. Scaffolds with free CF showed a burst release within 1 hour. The rate of AO release was dependent on the concentration at which the AO was blended into the scaffolds due to the segregation of the AO towards the surface of the fibres. This was believed to be driven by the ionic nature of the AO. HGF seeded on the scaffolds attached and had similar viability to those cells seeded on PCNU scaffolds without AO or CF; however, HGF showed significantly greater attachment on the CF scaffolds relative to the AO scaffolds. This interesting result was hypothesized to be due to the surface segregation of the CF or AO, leading to substantial differences in the surface chemistry for each scaffold. Scaffolds with a higher concentration of AO inhibited growth of characteristic microorganism \textit{P. gingivalis}.

The electrospun blend of PCNU with AO was further investigated in Chapter 4. The study outlined in this chapter also addresses \textbf{Objective 1}, with a focus on the effect of the AO and free CF on the biodegradation character of the PCNU nanofibres. The degradation profile is an important characteristic of a polymer scaffold, and the tunability of this parameter in synthetic polymers is one of the main advantages of using a synthetic material versus a natural polymer such as collagen or gelatin. \textbf{Chapter 4} and the corresponding paper (Wright et al. 2018) highlights the importance of investigating the effect of bioactive additives on scaffold degradation properties, as the CF and AO were found to have a significant effect on the rate of polymer matrix degradation. The thorough and quantitative methods that were applied allowed us to elucidate a mechanism by which the biodegradation rate of polymer scaffolds in general may be affected by the presence of these small molecular weight or oligomeric bio-additives. This is a landmark study as the field has devoted little attention to understanding the mechanism behind changes in biodegradation characteristics when introducing drugs into electrospun scaffolds, despite evidence that the addition of drugs alters the nanofibre degradation rate (Cui et al. 2006; Huang et al. 2006). Analytical characterization of the physical and chemical properties of the PCNU allowed us to elucidate the mechanism by which the AO and CF altered the degradation rate of the PCNU scaffolds (as determined by change in PCNU molecular weight via GPC). PCNU degradation rate is affected primarily by the amount of phase mixing of the PCNU hard and soft segments as well as hydrogen bonding of hydrolytically sensitive functional
groups; as such, by altering these chemical and physical properties, the AO and CF altered the rate of degradation of the PCNU matrix by increasing the hydrolytic stability of the PCNU. The combination of sustained drug release and delayed scaffold degradation presents a viable treatment modality for applications such as gingival tissue engineering that require long term antimicrobial management.

The studies outlined in Chapters 3 and 4 addressed the hypothesis associated with Objective 1 of the thesis. It reports on a novel use of the AO as a tool for attenuating the burst release commonly found when antibiotic is incorporated into nanofibres. The study finds significance within the field of electrospinning of drug in that the fibre diameter and alignment were kept constant and not allowed to be influenced by the changes in the composition of the scaffolds, thereby allowing us to directly assess the effects of drug form and loading on release properties, fibroblast cell attachment, and scaffold degradation. And while many authors have postulated on the phenomenon of drug surface segregation in blend electrospinning (K. Kim et al. 2004; Natu, de Sousa, and Gil 2010; He, Huang, and Han 2009), our study was the first in which surface localization was investigated as a function of drug loading using surface resolved XPS as a quantitative technique. Furthermore, the study’s intersection between the fields of tissue engineering and drug delivery allowed for the investigation of the effect of added drug on the cell adhesion character of the scaffold and scaffold degradation rate, which was found to be affected by the form and concentration of drug added and the propensity of the AO to surface locate.

The study reported in Chapter 5 addresses Objective 2 of the thesis. D-PHI was used as a scaffold platform for generating a prevascularized gingival construct using co-culture and perfusion flow culture. This study revealed the critical importance of flow rate as a means of controlling EC organization in a random-pore, synthetic scaffold. We demonstrated that a purely synthetic scaffold within which seeded cells undergo pro-angiogenic signalling pathways directed by ECM dynamics was shown to be directly affected by the flow conditions. Conditions at an intermediate flow rate (0.05 mL/min) generated a construct with an engineered microvessel network that successfully anastomosed with host vessels in a mouse subcutaneous model and produced greater neovascularization than a non-vascularized cell-seeded control. The significance lies in the proposal and validation of a method for prevascularizing a soft tissue
construct generated with D-PHI’s unique surface chemistry. Further, this approach does not rely on the incorporation of exogenous biomolecules within the scaffold to generate this result. Unlike the antimicrobial electrospun scaffold investigated in Chapters 3 and 4, it is a method toward generating an anti-infective construct that does not rely on the use of antimicrobial additives. To our knowledge, it is the first validated approach for prevascularization that does not rely on the use of bioactive macromolecules and/or organized pre-fabricated vessel channels within the as-made construct, and hence represents a method by which a prevascularized engineered tissue can be fabricated using materials and methods more favourably suitable for clinical translation. Furthermore, an advanced method of live imaging engineered vessels in situ is reported here wherein methods for ex vivo live tissue staining are applied in a subcutaneous implant model.

Two substantially different strategies toward generating an anti-infective gingival construct have been explored; however, both strategies are grounded in the concept of the “race to the surface” wherein bacteria may colonize a biomaterial implant and lead to implant-associated infection if the host’s own cells do not first attach and integrate into the implant (Gristina 1987). In the first strategy, release of antibiotic from electrospun antimicrobial scaffolds were shown to inhibit the growth of periodontal pathogen P. gingivalis without affecting the viability of attached HGF. As such, it can be used as a scaffold or construct wherein sufficient tissue integration can occur before bacterial attachment. In the second strategy, an engineered microvessel network was generated in D-PHI scaffolds in vitro such that when implanted in an in vivo model, the vessels anastomosed and became perfused with host blood. This would allow host immune defense mechanisms to act more immediately upon implantation of the construct.

The two strategies find similarities in that they both rely on synthetic polyurethane materials. These materials were fabricated with unique surface chemistries, geometries, and mechanical properties that allowed for control over their performance as anti-infective gingival constructs in terms of degradation, drug release, cell attachment, and tissue morphogenesis in in vitro models. Important findings related to cell-material interactions and scaffold material properties reported in this thesis will be valuable to many researchers working in the biomaterials field, particularly in biomaterials for drug delivery and tissue engineering. The specific strategies
investigated here may also be applied to other soft tissue regeneration where infection control is critical, such as in chronic wound treatment and skin substitutes for burn wounds.

6.2 Recommendations

The objectives of the thesis were met, and the hypotheses were tested adequately; however, some of the findings suggest the need for further studies toward generating a polyurethane-based anti-infective gingival tissue construct. The prevascularized construct can be recommended as a strategy for gingival tissue engineering with greater potential than the electrospun antimicrobial scaffold constructs as the construct proved to readily integrate and become perfused in a small animal model. It is recommended that further iterations in the design of the electrospun scaffold gingival constructs be investigated before the constructs are validated in an in vivo model. In particular, the relatively poor cell attachment observed for the AO scaffolds must be addressed, and the non-linear relationship between AO loading and release must be further investigated in order to elucidate an appropriate release profile for gingival tissue regeneration. Both the prevascularized constructs and the electrospun antimicrobial scaffold constructs must be tested in an in vitro and in vivo infection model that recapitulates the conditions of the gingival pocket. Future recommendations are proposed and outlined in greater detail below.

1. Further investigation of the dose-dependent release profile of AO in electrospun scaffolds (related to Objective 1).

This thesis demonstrated that the rate and extent of release for AO from the antimicrobial electrospun fibres was dependent on the concentration of AO that was blended into the fibres. As discussed in Chapters 3 and 4, the release profile of the 7 vs. 15% w/w CF-AO scaffolds differed in that almost 100% release of the AO was achieved within 7 days for the 15% w/w CF-AO scaffold, while release at day 7 for the 7% w/w CF-AO scaffold was still less than 50%. In electrospinning fabrication methods, blending a higher drug concentration can lead to the localization of drug at the surface of the fibres (K. Kim et al. 2004; Natu, de Sousa, and Gil 2010). A further study of the antimicrobial electrospun fibres in which other concentrations of AO between 7 and 15% w/w CF-AO may lead to the elucidation of a more ideal concentration that results in a release profile of AO that is more sustained throughout a physiologically relevant time period. Due to the significant difference in release profiles between the 7 and 15% w/w CF-
AO scaffolds, as well as the pronounced surface segregation effect demonstrated in the 15% w/w CF-AO scaffolds via surface-resolved XPS in Chapter 3, it is hypothesized that there is a critical concentration between 7 and 15% w/w CF-AO at which the tendency of the AO to localize on the outside of the electrospinning jet becomes pronounced. Investigating additional loadings of AO in the scaffolds may allow us to identify this critical concentration, as well as lead us to a formulation with a more ideal release profile. As proposed in the text in Chapter 3, a “sandwich” fabrication method in which fibres with varying concentrations of AO are layered in one mat could also be explored. Sandwich structures have been previously investigated in the electrospinning of drug in order to control the release profile of loaded drugs (Chen et al. 2012).

2. Investigation of the mechanism by which CF increased cell attachment (related to Objective 1).

An interesting finding in this thesis, presented in Chapter 3, is the phenomenon wherein fibroblasts seeded on scaffolds with free CF (15% w/w CF HCl) showed greater attachment than cells seeded on the AO scaffolds or PCNU scaffolds with no drug. Since the cells on scaffolds loaded with AO had similar viability to those on the 15% w/w free CF scaffolds (~90% viable), this effect was determined to be a cell attachment effect rather than a toxicity effect. It was hypothesized that surface segregation of the CF or AO lead to substantial differences in the surface chemistry for each scaffold, affecting protein attachment. The scaffolds with free CF would have presented a high number of carboxylic acid and amine functional groups, which are known to promote cell attachment and spreading (Faucheux et al. 2004), while the carboxylic acid of CF is not present in a high density for the AO scaffolds, or scaffolds with no additive. In addition to surface chemistry, electrostatic interactions between the scaffolds and absorbed proteins may also be playing a role in promoting and/or inhibiting cell attachment on the AO vs. free CF scaffolds (Schmidt, D.Waldeck and Kao 2009). Electrostatic charge on a biomaterial surface has been referred to as one of the main physical factors which determine the evolution of tissue on a material and has a strong effect on protein adsorption (K. Cai et al. 2006). In a preliminary study, we measured scaffold absorption of fibronectin, a key cell-adhesion protein (Appendix C). Interestingly, we found that the 15% w/w CF HCl scaffolds showed a significantly reduced concentration of fibronectin absorbed after 24 hours in a 20 µg/mL fibronectin solution. This finding prompts us to question further the mechanism by which the
free CF promoted HGF cell attachment on the scaffolds. Proposed future work would include measuring the surface charge on each scaffold type (zeta-potential). Future work may also investigate the conformation of absorbed fibronectin on the scaffolds, or the absorption character of other cell-adhesion proteins such as laminin and vitronectin (Anselme et al. 2010).

3. Mechanistic study of the critical effect of flow rate on EC organization in co-cultures on D-PHI in in vitro perfusion culture (related to Objective 2).

This thesis has established that in vitro perfusion flow can be used to promote the organization of ECs in a random-pore synthetic scaffold. The mechanism by which perfusion flow was able to enhance EC organization was concluded to be a combination of effects from shear forces that induced shear-mediated signalling pathways, as well as convective forces that controlled 3D gradients of pro-angiogenic signalling. It is recommended that the relative contribution of each of these forces be further investigated in future work, particularly for the intermediate flow rate of 0.05 mL/min which was used to produce the constructs studied in the in vivo model. In a preliminary experiment, growth medium from day 14 of the bioreactor culture period was collected and the relative concentration of various pro-angiogenic growth factors was measured using an antibody growth factor array. The results are reported in Appendix D. Soluble proteins associated with angiogenesis were elevated in the low and intermediate flow rates relative to the highest flow rate, which was attributed to the greater density of ECs at the low and intermediate flow rates. Further studies are required. It is recommended that the relative expression of pro-angiogenic growth factors associated with shear-mediated cell signalling be investigated via immunofluorescence staining of proteins embedded in the ECM or on cell-surfaces and/or Western blot and/or qPCR techniques. Convective forces are critical in controlling MMP production and spatial location, and MMPs are in turn critical for EC remodelling of the matrix to allow for angiogenesis (Davis, Bayless, and Mavila 2002). As such, staining of important MMPs, in particular MMP-9 (Cheng et al. 2011; Ciucurel, Vlahos, and Sefton 2014) and MMP-1 (Galie et al. 2014), may also help illuminate the mechanism by which flow was able to promote EC organization in the constructs. Finally, expression of key proteins in shear-mediated cell signalling pathways for both ECs and HGF can be measured. For example, Cheung et al. measured expression of focal adhesion protein β1-integrin as well as the level of focal adhesion kinase (FAK) phosphorylation in cultures of HGF on D-PHI under perfusion versus static culture.
and showed both were upregulated in perfusion culture, and that antibody inhibition of β1-integrin affected HGF phenotype in terms of production of the pro-contractile marker α-SMA (Cheung et al., 2014). It is recommended that a similar experiment be completed with co-cultures of HGF and HUVECs on D-PHI under perfusion in order to assess morphological changes in the constructs when shear-mediated signalling pathways are blocked or inhibited. This will help to elucidate relative contributions of shear- versus convective-based forces in flow-mediated promotion of EC organization in the constructs.

Additionally, an experiment may be completed in which the flow rate is maintained while the viscosity of the growth medium is altered through the addition of a thickening agent such as dextran; growth medium with 6% dextran (~70,000 g/mol) has a viscosity that is about three times greater than normal growth medium (Sikavitsas et al. 2003). According to the estimation of shear stress model used in this thesis (Chapter 5.7.1), increasing the viscosity of the growth medium by three times would result in shear stresses approximately three times greater, without influencing convective forces in the constructs. However, there are significant drawbacks to this approach, for example increasing viscosity with 6% dextran may not result in a great enough increase in shear stress magnitude experienced by cells, and increasing the concentration of dextran in the medium any greater than 6% may produce a cytotoxic effect (Li, Dai, and Tang 2008).

4. Investigation of temporal-based (dynamic) flow rate magnitudes for perfusion co-cultures on D-PHI (related to Objective 2).

This thesis reported that the high flow rate magnitude of 0.5 mL/min led to the shearing off of a significantly greater number of cells in the first 3 days of culture, and it was hypothesized that the majority of the cells sheared were ECs (Chapter 5). HUVECs have been shown to be more sensitive to shearing from biomaterial scaffolds at early time points relative to fibroblasts (Feugier et al. 2005). So while it was hypothesized that high flow rates would activate shear-mediated cell signalling pathways and promote faster and/or more extensive vessel organization (Kang, Bayless, and Kaunas 2008; Cheung et al. 2015), it is likely that the high flow used in this thesis was too high and too soon to have any beneficial effect on EC organization into lumens. It is proposed that an additional study be completed in which low flow rates, similar in magnitude
to the 0.005 and 0.05 mL/min flows, be used initially during the perfusion culture for a period of ~7 days, and then the flow be ramped up to the higher magnitude. Using a higher magnitude flow once the cells have produced adequate ECM tissue and demonstrated strong attachment would impart shear stresses on the cells at a magnitude that is known to activate shear-mediated signalling pathways for EC organization (Kaunas, Kang, and Bayless 2011) without compromising EC integrity (Buchanan et al. 2014). Dynamic flow has previously been used in perfusion culture studies as a means of optimizing cell proliferation along with cell phenotype and behavior (McCoy, Brien, and O’Brien 2010). In such a study, a dampener in the flow loop may be used to ensure that the flow of growth medium in non-pulsatile. Some studies have shown a different EC response to flows that are pulsatile vs. non-pulsatile (Abe et al. 2012).

5. Investigation of potential synergistic effect of hypoxia and perfusion flow in in vitro culture (related to Objective 2).

Hypoxia is a powerful driver of angiogenesis (Lamalice, Le Boeuf, and Huot 2007; Davies et al. 2011), and hypoxia-induced signalling pathways have previously been cited as governing mechanisms behind angiogenic sprouting in 3D scaffolds under perfusion flow (Malda, Klein, and Upton 2007; Rouwkema and Khademhosseini 2016; Landau, Guo, and Levenberg 2018). In the current thesis, it was hypothesized that hypoxia at the low flow condition (0.005 mL/min) promoted the organization of ECs in the gingival constructs (Chapter 5). It is recommended that in a future study, the pro-angiogenic effects of both hypoxia and perfusion flow be utilized by carrying out the in vitro perfusion culture in a hypoxic environment (i.e. in a tri-gas cell culture incubator to control oxygen tension). In such a set-up, it would be possible to submit the HGF and the HUVECs seeded in the construct to higher flow rates to improve convection of wastes, nutrients, and signalling molecules through the construct as well as impart shear stress, while also activating hypoxia-induced angiogenesis signalling pathways. Furthermore, hypoxic conditions have been shown to upregulate expression of pro-angiogenic growth factor TGF-β1 (Falanga et al. 1991). It was later shown that despite increased expression of TGF-β1, which is known to promote fibrosis in tissues, hypoxia significantly reduced myofibroblast differentiation as measured by the production of the contractive protein α-SMA (Modarressi et al. 2010). Therefore, co-culture of HGF and HUVECs may benefit greatly from hypoxic conditions in vitro in terms of tissue development and EC organization.
6. Validation of anti-infective character in an in vivo infection model (related to Objectives 1 and 2).

An important future study will validate the anti-infective quality of the gingival tissue engineering scaffold platforms in an in vivo setting. While it was shown that the CF incorporated into the electrospun antimicrobial scaffolds was bioactive against the periodontal pathogen *P. gingivalis*, and that engineered microvessels in the prevascularized constructs anastomosed with host vessels within 14 days in an in vivo mouse model, it has yet to be proven that the platforms can successfully favour tissue integration over bacterial colonization when implanted in an infectious environment. For such a study, an in vivo model must be designed as there is no widely accepted model for tissue engineering in an infectious environment. There is also no simple and reproducible model that adequately mimics clinical pathogenesis of periodontal disease (Oz and Puleo 2011). Non-human primates, dogs, and ferrets all have sufficiently similar oral environments to humans in terms of molar teeth structures as well as gingivitis and periodontitis pathogenesis, but the expense associated with the use of these animals limit their feasibility as animal models for the proposed study (Oz and Puleo 2011; Struillou et al. 2010). A rabbit model has been used for investigating an antibiotic-loaded scaffold for craniofacial tissue regeneration in which bacterial pathogens were inoculated (Shah et al. 2016). This model may be adapted for the current purpose wherein we would assess functional gingival tissue regeneration after pathogen inoculation. Periodontal disease can be induced in rodents similarly by inoculation, or via placement of a bacterial retentive silk/cotton ligature around the molar teeth (Rajshankar et al. 2013; Oz and Puleo 2011; Struillou et al. 2010). In such a model, an ibuprofen-loaded electrospun scaffold has been shown to increase gingival attachment (Batool et al. 2018). However, the rodent oral environment is miniscule which makes oral surgery and tissue harvesting a challenge. It is recommended that in future steps, an established subcutaneous infection model in a rodent be used in place of an oral in vivo model (Gilchrist et al., 2013b; Lan et al., 2014; Rozenbaum et al., 2019). It would not be possible to assess functional regeneration (i.e. gingival attachment), but the ability of the scaffolds to successfully integrate into the host tissue in a timely manner relative to controls without anti-infective capabilities could be assessed in a more practical manner. Alternatively, more complex in vitro models may be used such as that developed by Bao et al. in which a perfusion bioreactor system was used as an oral infection model resembling the periodontal pocket (Bao et al. 2015; Shang et al. 2018).
References


Dawson, Deborah V, David R Drake, Jennifer R Hill, Kim A Brogden, Carol L Fischer, and Philip W Wertz. 2014. “Organization, Barrier Function and Antimicrobial Lipids of the


Galie, Peter A, Duc-Huy T Nguyen, Colin K Choi, Daniel M Cohen, Paul a Janmey, and Christopher S Chen. 2014. “Fluid Shear Stress Threshold Regulates Angiogenic Sprouting.”


Kaunas, Roland, Hojin Kang, and Kayla J Bayless. 2011. “Synergistic Regulation of Angiogenic Sprouting by Biochemical Factors and Wall Shear Stress.” *Cellular and Molecular*


Koch, M A, E J Vrij, E Engel, J a Planell, and D Lacroix. 2010. “Perfusion Cell Seeding on


Lamba, NMK, KA Woodhouse, and SL Cooper. 1998. *Polyurethanes in Biomedical Applications*. Boca Raton, FL: CRC.


McBane, Joanne E., Soroor Sharifpoor, Kuihua Cai, Rosalind S. Labow, and J. Paul Santerre. 2011. “Biodegradation and in Vivo Biocompatibility of a Degradable,


doi:10.1016/j.biomaterials.2015.10.026.


149


Pham, Quynh P, Upma Sharma, Ph D Antonios G Mikos, and D Ph. 2006. “Electrospinning of Polymeric Nanofibers for Tissue Engineering Applications : A Review” 12 (5).


Toncheva, Antoniya, Dilyana Paneva, Vera Maximova, Nevena Manolova, and Iliya Rashkov. 2012. “Antibacterial Fluoroquinolone Antibiotic-Containing Fibrous Materials from poly(L-


Appendices

A. Controlled Release of Antibiotic from Synthetic Porous Scaffolds for Application in Gingival Tissue Engineering

In this appendix chapter we report a short study wherein a third anti-infective scaffold strategy was conceived and investigated. This study does not address the main objectives of the thesis but rather builds on concepts developed in each of the two main objectives in order to propose a scaffold platform that married the antimicrobial capacity of the electrospun PCNU with the mechanical and chemical properties of the D-PHI scaffold, herein referred to as CF-DPHI.

Highlights:

- An antimicrobial oligomer conceived by Delaviz et al. containing a pendant CF-PEG chain (Delaviz et al. 2015) was incorporated into D-PHI scaffolds at concentrations corresponding to 3 and 6wt% CF.
- Release of CF from CF-DPHI was sustained over 28 days and followed zero-order kinetics.
- HGF seeded on the 6wt% CF-D-PHI scaffolds showed a greater cellularity at day 14 compared to at day 7.

This study was presented at the Society for Biomaterials annual meeting in Minneapolis in 2017: Author Y. Delaviz provided the antimicrobial oligomer and carried out the mechanical testing of the scaffolds as well as their gel content and swell ratio.

Abstract
Synthetic scaffolds may be used to generate tissue engineered constructs for the regeneration of gingival connective tissues that are degraded during the progression of periodontal disease. However, the use of a synthetic material in the infectious oral environment has the potential to lead to a biomaterial-associated infection. The objective of the current study was to incorporate a ciprofloxacin (CF) based divinyl monomer (HLHCFPEG) into a cross-linked degradable polyurethane hydrogel, D-PHI, and to investigate the rate of CF release and the effect of the added drug on the mechanical and cell compatibility properties of the scaffolds. The novel CF-DPHI scaffolds had a final CF concentration of 3 and 6 wt%. Release of CF from CF-DPHI incubated under static conditions in cell growth medium at 37°C was sustained over 28 days and followed zero-order kinetics. 6wt% CF-DPHI did not produce 2x the CF release as anticipated, rather there was no significant difference in release for the 3 vs. 6wt% CF-DPHI. The swell ratio of the scaffolds when incubated in distilled water was also determined, and it was found that both 3 and 6wt% CF-DPHI have significantly lower swell properties than DPHI, and that 6wt% has a slightly lower swell ratio than 3wt% CF-DPHI. It is hypothesized that CF release from the 6wt% CF-DPHI scaffolds was reduced due to possible loss of unreacted CF monomer during the polymer preparation, and/or reduced water uptake leading to less CF diffusion and potentially less hydrolysis-dependent release. The addition of HLHCFPEG did not alter the cell compatibility of the scaffolds. In summary, the novel CF-DPHI scaffold platform produced a controlled and sustained release of antibiotic that was compatible to gingival cells. A slow and sustained release of antibiotic to the local area during periodontal tissue regeneration may prevent infection-related failure in tissue engineered grafts.
A.1 Introduction

Synthetic scaffolds may be used to generate tissue engineered constructs for the regeneration of gingival connective tissues that are degraded during the progression of periodontal disease. However, the use of a synthetic material in the infectious oral environment has the potential to lead to a biomaterial-associated infection (Marco C. Bottino et al. 2012). Antimicrobial polymers have previously been explored as a means to deliver drug in a sustained and controlled manner (G. L. Y. L. Woo, Mittelman, and Santerre 2000). The broad-spectrum antibiotic ciprofloxacin (CF) was incorporated into a degradable polyurethane; however, incorporating CF into the polymer backbone via covalent linkages resulted in a number of potentially bioactive degradation by-products that could not be easily characterized. As such, an antimicrobial oligomer (AO) was synthesized and incorporated into a degradable polycarbonate polyurethane (PCNU) nanofibre scaffold via blend electrospinning such that the only bioactive degradation product was CF (Wright et al. 2018, 2017). The AO was hydrolysed to release CF in a sustained manner. While CF was released from the AO in a linear release profile, diffusion of the AO from the fibres displayed a burst release profile, especially when the AO was blended in the fibres at a higher concentration (Wright et al. 2018, 2017). While the aforementioned work by Wright et al. showed that incorporating CF as part of the AO improved the distribution of the drug within the fibres, the studies did not demonstrate that the majority share of the AO released from the fibres within 7 days would remain in situ and prevent colonization of the scaffold for the duration of tissue integration of the scaffold (Wright et al. 2017).

With that in mind, an approach in which CF was covalently cross-linked into a gingival tissue engineering scaffold was explored. The CF was incorporated into the cross-linked polymer scaffold D-PHI via a novel cross-linking divinyloligomer (DVO) recently reported on for applications in dental adhesives by Delaviz et al (Delaviz, Nascimento, et al. 2018). D-PHI is a degradable polar hydrophobic ionic hydrogel scaffold with a di-vinyl urethane-based oligomer called DVO (divinyl oligomer). D-PHI and DVO are non-commercial materials invented and synthesized in-house (Sharifpoor, Labow, and Santerre 2009). D-PHI has shown promise as a scaffold for gingival tissue engineering (Cheung et al. 2015) as well as vascular tissue engineering (K G Battiston et al. 2015), and has a proven immunomodulatory surface chemistry that pushes monocytes toward a more wound-healing phenotype (Battiston et al. 2015). It was
therefore of interest to give antimicrobial functionality to D-PHI scaffolds to improve their usefulness for soft tissue engineering applications in infective environments such as the oral environment. The objective of the current study was to incorporate the CF-based divinyl monomer into D-PHI, and to investigate the in vitro rate of CF release from the scaffold and the effect of the added drug on the mechanical and cell compatibility properties of the scaffolds.

A.2 Materials and Methods

Degradable, polar, hydrophobic and ionic (D-PHI) synthetic hydrogel scaffolds were fabricated via free radical polymerization as per methods reported elsewhere (Cheung, Rose, and Santerre 2013), but modified with the incorporation of a divinyl CF-based monomer resulting in a novel scaffold material with a final CF concentration of 3 and 6 wt%. The CF monomer, previously reported on in detail elsewhere (Delaviz, Nascimento, et al. 2018), is known as HLH-CFPEG. HLH-CFPEG consists of an oligomeric backbone of two molecules of hydroxyethyl methacrylate (HEMA) linked via urethane linkages to lysine diisocyanate (LDI). Polyethylene glycol (PEG, molecular weight ~340 g/mol) functionalized with CF was then covalently bonded to the HEMA-LDI-HEMA backbone. The pendant CF is linked via an amide bond that is readily hydrolysed to release CF-PEG and free CF.

D-PHI as well as 3 and 6wt% CF-D-PHI scaffolds were incubated under static conditions in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum at 37°C for 28 days and CF release was measured over time using high performance liquid chromatography (HPLC). Scaffold physical properties were investigated for gel content quantification, swell ratio determination, and compressive mechanical testing. Metabolic activity (WST-1 assay) and DNA content (Hoechst fluorescence intensity assay) of human gingival fibroblasts (HGF) seeded on the scaffolds were measured after 7 and 14 days. CF-D-PHI scaffolds were imaged using confocal microscopy, wherein the CF autofluoresces such that the distribution of CF in the scaffolds can be seen.

Unless otherwise stated, all experiments were repeated in duplicate (N=2) with n=3 samples per repeat (n=6 in total). A one-way ANOVA was used to determine statistically significant differences between groups (the significance threshold was set to $\alpha = 0.05$). When statistical significance was found, Tukey’s HSD post hoc analysis was used to determine which
of the groups contributed to the statistical significance. Levine’s test of homogeneity of the variances was conducted.

A.3 Results and Discussion

It was found that there was a uniform distribution of fluorescent signal from the CF throughout the 3 and 6wt% CF DPHI scaffolds, and the intensity of the signal was stronger in the 6wt% scaffolds, indicating a greater concentration of CF in these scaffolds (Figure A-1).

Figure A-1. Cross-sectional images of CF-DPHI with cross-linked HLH-CFPEG. The CF in the HLH-CFPEG autofluoresces and is shown in green. All scale bars = 100µm.

Release of CF from CF-DPHI was sustained over 28 days and followed zero-order kinetics (Figure A-2). 6wt% CF-DPHI did not produce 2x the CF release as anticipated, rather there was no significant difference in release for the 3 vs. 6wt% CF-DPHI, suggesting other possible controlling phenomena within this in vitro system, independent of CF concentration.

Figure A-2. Cumulative release of CF in μg/mL from CF-DPHI scaffolds loaded with HLH-CFPEG in DMEM growth medium at 37°C. Data are mean ± SD (n=6).
Gel content was measured to determine the cross-linking efficiency of the scaffolds. While all scaffolds had high conversion (>90% gel content), the scaffolds with the highest amount of CF had a significantly lower gel content (Figure A-3). Therefore, some of the CF monomer may have been lost during the monomer leaching step which models pore formation in the scaffolds, post synthesis. The swell ratio of the scaffolds when incubated in distilled water was also determined, and it was found that both 3 and 6wt% CFDPHI have significantly lower swell properties than DPHI, and that 6wt% has a slightly lower swell ratio than 3wt% CF-DPHI (Figure A-3). It is hypothesized that CF release from the 6wt% CFDPHI scaffolds was reduced due to possible loss of unreacted CF monomer during the polymer preparation, and/or reduced water uptake leading to less CF diffusion and potentially less hydrolysis-dependent release. There was no significant difference in the compressive elastic modulus for DPHI vs. CF-DPHI scaffolds (Figure A-4).

Figure A-3. The gel content (%) (A), and the swell ratio (% change in mass) of DPHI and CF-DPHI scaffolds soaked in water for 5 days (B) of the corresponding polymerized materials. * represents significant difference from other groups (p<0.05). Data are mean ± SE (n=5).
Figure A-4. Compressive elastic modulus for varying wt% CF incorporated. Data are mean ± SD (n=6).

There was no significant difference in the metabolic activity (WST-1) of HGF seeded on the CF-D PHI scaffolds compared to 0wt% CF controls after 7 and 14 days of culture (Figure A-5). HGF seeded on the 6wt% CF-D PHI scaffolds showed a greater cellularity at day 14 compared to at day 7 (Figure A-5). Incorporation of a CF oligomer into gingival tissue engineering scaffolds has previously been shown to promote HGF attachment (Wright et al. 2017). The antimicrobial efficacy of the scaffolds must be tested against the periodontal pathogen Porphyromonas gingivalis.

Figure A-5. HGF metabolic activity (WST-1 absorbance) and DNA content (ng of DNA) after 7 and 14 days in static culture. Data are mean ± SD (n=6). * significant difference (t-test, p<0.05).
A.4 Conclusion

The current work shows the use of a unique polymer scaffold that incorporates CF for soft tissue engineering in an infectious environment. A novel divinyl CF monomer was used in a synthetic hydrogel to produce a controlled and sustained release of antibiotic that was compatible to gingival cells and would minimally alter the mechanical properties of the scaffolds. A slow and sustained release of antibiotic to the local area during periodontal tissue regeneration may prevent infection-related failure in tissue-engineered grafts. Future work is required to assess the antimicrobial efficacy of the scaffolds.

Acknowledgements

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Abstract: The Biomedical Polymers Laboratory at the Institute of Biomaterials and Biomedical Engineering in Toronto in the Faculties of Applied Science and Engineering, Dentistry and Medicine is coordinated by Professor J Paul Santerre in the Institute’s Biomaterials, Tissue engineering and Regenerative Medicine theme area. Research in the Santerre group is focused on new polymer synthesis and specifically polyurethane chemistry for biomedical implants and their bio-stability, conceiving new biomaterials for tissue engineering applications of vascular and connective tissues, and utilizing co-culture techniques as a means of circumventing complicated biochemical modifications of materials. In this reported work, D-PHI interactions in both two and three dimensional architectures with human gingival fibroblast (HGFs) and endothelial cells (ECs) show good cell growth, viability and collagen production relative to established degradable biomaterials such as polylactic-glycolic acid (PLGA) and tissue culture materials such as tissue culture polystyrene (TCPS). In addition, HGFs cultured on D-PHI show VEGF production under optimal perfusion flow conditions, thereby enabling the potential to contribute to micro-vessel formation when the materials are applied towards the development of gingival tissues. The work further shows the potential use of D-PHI as an elastomer under cyclic mechanical loading for vascular tissue engineering applications, as indicated by the material’s ability to support an anti-inflammatory monocyte state, which combine to also support the growth and modulated contractile phenotype of vascular smooth muscle cells (VSMC).

C. Fibronectin Absorption Characteristics on Antimicrobial Electrospun Scaffolds

A 20 μg/ml fibronectin solution was prepared by diluting a 12.5 μg/ml fibronectin solution (Centre de Transfusion Sanguine des Armées, France) in PBS (-/ -). Four 6 mm scaffold samples (0%, 7%, and 15% w/w CF (AO) as well as 15% w/w free CF) were placed in separate wells in a 96 well polystyrene plate. 150 μl of the 20 μg/ml fibronectin solution was added to three samples of each scaffold type. 150 μl of PBS was added to the remaining samples to serve as a negative control. The samples were incubated at 37°C for 24 hours. All samples were then washed twice with PBS before incubation in 250 μl of a 2% sodium dodecylsulphate (SDS) solution on a shaker plate overnight. The scaffolds were then removed from the SDS solutions and a bicinchoninic acid (BCA) assay (Life Technologies) was applied using albumin standard controls. The absorbance at wavelength 562nm of all samples and standards were read with a plate reader and an absorbance versus protein concentration curve was created by the standard control values. The protein concentration of the samples was quantified by the standards curve and the fibronectin mass per surface area of scaffold was calculated. The results are shown in Figure C-1. There was no significant difference between the 0% w/w CF scaffolds and any other scaffold type, however the 15% w/w free CF scaffolds adsorbed significantly less fibronectin than the scaffolds which contained the AO.
Figure C-1. Protein adsorbed onto scaffolds after samples were incubated for 24 hours in a 20μg/ml fibronectin solution.

Fibronectin conjugated in-house with Alexa Flour® 568 fluorescent antibody (ThermoFisher Scientific) was diluted to 20 μg/ml in PBS(−/−). This fluorinated fibronectin solution was used in the process described in place of native fibronectin. Following the 24 hour incubation period, samples were washed three times in PBS and imaged using a fluorescent microscope. The fibronectin appears red in the images and illustrates the distribution of the fibronectin on the surface of the scaffolds (Figure C-2). There is some autofluorescence of the scaffolds, likely due to the ciprofloxacin in the fibres (Figure C-3). While the 0% CF scaffolds and AO scaffolds appear to have a uniform fluorescence intensity across the scaffold surface, the 15% w/w free CF scaffold appear to have localized areas of higher intensity fluorescence.
Figure C-2. Images of fluorescent fibronectin adsorption on scaffolds after samples were incubated for 48 hours in a 20 μg/ml fluorescent fibronectin solution. Scale bar = 130 µm.

Figure C-3. Images of autofluorescence from scaffolds after samples were incubated for 24 hours in PBS.
D. Quantification of Soluble Pro-Angiogenic Growth Factors Present in Perfusion Bioreactor Growth Medium

As discussed in Chapter 5, the mechanism by which the perfusion flow rate produced a critical effect on the tendency of the ECs to organize into lumen structures is complex. Cell signalling via growth factors plays an important role. It was hypothesized that shear forces produced by medium perfusion may result in the upregulation of shear-mediated pro-angiogenic growth factors, particularly for the cells subjected to 0.05 and 0.5 mL/min flow. It was also hypothesized that hypoxic conditions at the low flow rate may result in hypoxia-induced upregulation of pro-angiogenic proteins. Growth factors in both soluble and matrix-embedded form may exert effects on EC signalling. In the study reported here, the relative concentration of soluble pro-angiogenic growth factors and other angiogenesis-related proteins from bioreactor growth medium were measured via a protein array. The array was performed on the growth medium collected from the bioreactors after the last 48 hours of culture. The results are shown as a heat map in
Table D-1.

In general, soluble proteins associated with angiogenesis were elevated in the low and intermediate flow rates relative to the highest flow rate, contrary to the hypothesis that high shear conditions would result in a greater concentration of soluble angiogenesis-associated proteins in the growth medium. It is suspected that there is a lower concentration of angiogenesis-associated proteins in the high flow rate condition due to the lower density of ECs at this condition that was determined previously via IF imaging. Many of the proteins upregulated in hypoxic conditions produced a greater fluorescence intensity in the low flow rate condition, which is suggestive of the existence of a hypoxic condition in the constructs at the low flow rate (Table D-2A). However, mechanosensitive cell surface receptors were also upregulated in the lower flow conditions relative to the high flow conditions (Table D-2B). We concluded that the growth factor array was not an adequate means of investigating cell signalling pathways in the constructs at each flow rate, and that further studies were required.
Table D-1. Angiogenesis-associated soluble proteins measured as fluorescence intensity with a heat map applied between flow rates for each protein (red = high) (N=3). The proteins were further arranged according to whether they have been shown in the literature to promote, stabilize, or inhibit vessel organization.

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Table D-2. (A) Angiogenesis-associated soluble proteins shown to be upregulated under hypoxic conditions (Krock, Skuli, and Simon 2011) and (B) Mechanosensitive cell surface proteins (Garin and Berk 2006). Measured as fluorescence intensity with a heat map applied between flow rates for each protein (red = high) (N=3). The proteins were further arranged according to whether they have been shown in the literature to promote (“Prom”), stabilize (“Stab”) or inhibit (“Inhib”) vessel organization.

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