Controlling CO Dose from CORM-loaded Electrospun Scaffolds with Diffusion-based Modeling and Experimental Assessment of Endothelial Cell Response

by

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Abstract

Title
Controlling CO Dose from CORM-loaded Electrospun Scaffolds with Diffusion-based Modeling and Experimental Assessment of Endothelial Cell Response

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Cardiovascular disease (CVD) accounts for one in every five deaths in the United States alone due to occlusion in small diameter (< 6 mm) vessels. The current treatment options includes bypass grafting; however, more than 30 percent of patients do not have viable saphenous veins for autologous grafting [1]. Therefore, tissue engineering is being considered as an alternative approach. The overall goals of this project were to develop a tissue engineered vascular graft with the incorporation of carbon monoxide releasing molecules (CORMs) and to determine the impacts of carbon monoxide (CO) on endothelial cells (ECs) with the goal of promoting a functional endothelium within the graft. This was accomplished through two complementary studies: investigating the impacts of CO-loaded electrospun scaffolds on ECs for cardiovascular applications and diffusion-based modeling of drug delivery of gasotransmitters from tissue engineered scaffolds. In the first study, we extended the maximum in vitro incubation time to permit better cellular attachment and proliferation with a newly-synthesized, more hydrophobic CORM (DK3) and established the impact of CORMs on EC viability and function (e.g. reactive oxygen species (ROS) products, and ROS levels). We further investigated
toxicity and biocompatibility of a newly synthesized CORM (DK4) loaded within PCL thin films and nanoparticles. We concluded that the DK4 and other compounds are not toxic at doses ranging from 0 – 50 µg/mL to the ECs both within nanoparticles that can be internalized within cells. We also conducted an in vivo pilot study to determine graft biocompatibility and preliminary results showed that CORM implants maintain mechanical integrity, support blood flow, and do not show toxicity for up to six weeks. In the second study, we validated a computational model and analyzed the output of CO delivery to better understand and control local dose. For CO release, this model is necessary because of the limitations with real-time experimental analysis and the need to better understand the dose available to the cells. We demonstrated that the validated model can be used to predict drug availability to cells for a variety of scaffolds and drug molecules. Our simulated results suggest that only a fraction of the initial concentration of gasotransmitters released from fibers that enters the interstitial fluid in vivo, or culture media in vitro, will be available to cells. We also demonstrated that fiber orientation and fiber diameter are important for drug delivery, but fiber density provides even more important information. The more contact area within the fiber scaffolds is equivalent to experimental conditions with more cell attachment and spreading. These parameters are not only important for traditional tissue engineering, but also for drug delivery. Overall, these results demonstrate the feasibility of making a tissue engineered vascular graft with the incorporation of CORMs and validating the importance of computational modeling of diffusion-based transport of CO. Future
work will involve performing surface modifications to enhance cell attachment and proliferation, using the developed computational model to better predict dose available to cells, and experimentally determining impact of CO dose on ECs.
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Dedicated to the memory of my Godmother,

Mrs. Gail Marie Carr Martin
Chapter 1: Introduction

1.0 Introduction

Cardiovascular diseases (CVDs) are a collection of diseases including coronary artery disease, angina, carotid artery disease, and peripheral artery disease (PAD). CVD is the reason for one in every five deaths in the United States alone [2]. Some CVDs are a result of thrombosis or atherosclerosis, which is composed of cholesterol, fatty substances, cellular waste products, calcium, and fibrin buildup in the arteries. Occlusion of small diameter vessels is the most common cause of CVD. The current gold standard for treatment is bypass grafting of autologous vessels from the patient, but greater than 30% of patients lack viable saphenous veins for autologous grafting because of previous harvest, systematic vascular disease or due to insufficient size (e.g. length and/or diameter) [1]. Research groups have investigated the use of non-degradable synthetic prostheses utilizing expanded poly(tetrafluoroethylene) (ePTFE) or Dacron (polyethylene terephthalate) for graft material for small diameter applications; however, these clinical grafts demonstrate limited endothelial function and fail due to intimal hyperplasia and thrombosis for this application [3]. Therefore, it is necessary to develop a therapeutic molecule loaded-tissue engineered graft that promotes self-repair as an alternative to generate a functional tissue engineered vascular graft (TEVG).

Current tissue engineering approaches for treating CVD involves fabricating scaffolds to promote or improve blood flow through the diseased artery and stimulate tissue regeneration. Studies have reported the ability to produce small diameter grafts
and maintain graft integrity up to 4 months, but the attempts to develop small
diameter TEVGs less than 4 mm have not been successful [4]. The extended time
period of survival is prohibited by stenosis and intimal hyperplasia, which could
benefit from improved endothelialization [5–7]. In this study, we developed a
vascular graft loaded with a therapeutic molecule that can provide local delivery into
a diseased site and investigated the impacts of CO on vascular cell types, with ECs
in particular.

1.1 Cardiovascular Disease

Myocardial infarction, peripheral artery disorders, and coronary artery
occlusion all make-up CVD [2,8]. These diseases, together, are one of the main
causes of death worldwide. Approximately 92.1 million American adults have one
or more types of CVD, and it is also predicted that 43.9% of the United States
population will have some form of the disease by 2030 [8]. To address this problem,
it is important to understand the native and diseased tissues.

1.1.2 Native tissue

Arteries contain three distinct layers that contribute to the structure and
function of the vascular system. Figure 1A illustrates the morphological layers,
which include the intima (the inner most layer made of endothelial cells), the media
(the middle layer consisting of SMCs, collagen, and elastin), and the adventitia
(composed of fibroblasts, collagen and glycosaminoglycans) [9,10]. Arteries are
responsible for transporting blood away from the heart as shown in Figure 1B and
their thick walls are able to withstand high blood pressure passed from the heart. However, atherosclerosis can cause the narrowing of arteries as a result of the buildup of atherosclerotic plaque (containing lipids, cholesterol, fats and cellular debris) (Figure 1C). This limits the flow of oxygen-rich blood throughout the body, which leads to the risks of severe conditions in coronary artery or peripheral artery disease, and warrants treatment or surgery.

**Structure of an Artery Wall**

*Figure 1. A) Illustrates the morphology of the artery wall, B) demonstrates normal blood flow, and C) represents the build-up of atherosclerotic plaques in the artery.*

Labeled for reuse at https://www.researchgate.net/figure/Layers-of-an-artery_fig1_304000395
https://www.flickr.com/photos/healthaliciousness/20742841461
1.1.3 Current Treatment for Cardiovascular Diseases

Current possible surgical treatment options are angioplasty or bypass graft surgery by using autologous vascular grafts or synthetic polymer grafts [11]. However, when the occlusion is severe or is in multiple locations, bypass grafting is recommended for treatment.

Bypass grafting is a surgical procedure that uses grafts to reroute blood flow around the restricted arteries. This method requires more than one graft depending on the severity of the patient’s narrowed arteries. The three currently used options are allogenic, autologous and synthetic grafts for treatment. Allografts or xenografts are grafts from different species, but these types of grafts have possible concerns of immune rejection and disease transmission [12]. Autologous grafting uses the internal mammary artery or saphenous vein from the patient. Even though this type of graft is the gold standard, more than 30% of patients do not have viable supply for grafting [1]. In addition, autologous grafts are still prone to atherosclerosis and intimal hyperplasia even with successful grafting [3]. The use of non-degradable synthetic grafts is the third option. These grafts are made of expanded polytetrafluoroethylene (ePTFE) or polyethylene terephthalate (PET) (Dacron), and are used as clinical products for successful grafts in large diameter vessels (6-10 mm). The challenge is presented in small diameter vessels (<6 mm) because they typically develop occlusion and thrombosis [13–15]. Therefore, generating TEVGs for vascular applications has risen to the forefront of research goals in many groups.
1.2 Tissue Engineering

The field of tissue engineering has continued to rapidly develop since the term was coined at a National Science Foundation workshop in 1988 [16], and it is expected to evolve steadily in the future. Tissue engineering originated from multiple fields including the life sciences and several disciplines of engineering. The subject involves the use of scientific and engineering principles to incorporate living cells along with a biodegradable scaffold to develop biological substitutes for implantation into the body that can induce a tissue regeneration response through extracellular matrix (ECM) deposition and maturation [17]. Figure 1.2 shows a diagram of a typical tissue engineering strategy or triad that acts as a model for tissue-engineered research.

Figure 1.2. Tissue Engineering Strategy that integrates cells, scaffold, and growth factors to promote tissue regeneration.
The model demonstrates components used within a tissue engineered system that are often designed to closely mimic the native environment of the desired tissue. The components include scaffolds, cells and growth factors, and bioreactors [16]. Each component of this model serves as an integral part in promoting reconstruction of tissue, in addition to improving the overall structure and function of the native tissue. These will be discussed in the next three sections.

1.2.1 Scaffold

The field of tissue engineering utilizes 3D porous scaffolds to offer the proper parameters for promoting cell attachment, migration, and proliferation to mimic the native tissue and organ [16]. Scaffolds are very important to the tissue engineered system because they supply the initial mechanical structure and the initial binding site for the cellular component [18]. The scaffold should have mechanical properties similar to the anatomical site implanted. The scaffold must also have specific properties including being biocompatible and biodegradable over time [16]. Biocompatibility is the initial requirement for any tissue engineered scaffold because cells are expected to adhere, migrate, and function as closely as they would in the native vessel [16]. In addition, biocompatibility, after implantation, is essential because the scaffold should prompt an inflammatory response to prevent a severe inflammatory response causing rejection in the body [16]. An aim of tissue
engineering is to allow autologous cells from the body to regenerate or replace the implanted conduit. Therefore, TEVGs are not permanent implants, and they must be biodegradable. The scaffold should degrade so that the cells can replace the scaffold with new ECM, and the degradation products must not be toxic to the tissue or to the body [16]. Scaffold properties including surface characteristics, porosity, and mechanical stability are important factors to be considered.

1.2.2 Growth factors, Bioreactors, and Therapeutic molecules

The incorporation of growth factors, bioreactors, and/or therapeutic molecules for tissue engineering systems are commonly used strategies. Cells can recognize scaffolds by integrating soluble bioactive molecules, such as growth factors and therapeutic molecules, within the biomaterial to modulate tissue formation [19–23]. However, for tissue engineering strategies, bioreactors are extremely important because they provide an in vitro condition with aspects of an in vivo environment for tissue regeneration. Bioreactors also allow the systematic analysis of cell response to external cues similar to that of the native tissue [24]. Using bioreactors in a tissue engineering strategy assists primary cells to maintain their phenotype through biophysical, mechanical, and biochemical cues. Particular approaches include use of bioreactors to promote cell attachment and growth into a porous scaffold prior to implantation. Therapeutic molecules and their impact on cells are also important to the design of this bioreactor system.
1.2.3 Cellular Components

The design of TEVGs with proper mechanical structure and function, biocompatibility, and degradation characteristics is important; however, the ability to develop a biomimetic TEVG for cardiovascular applications strongly rely on considering aspects of the anatomical structure and biological function of the blood vessel [23]. As previously mentioned in Section 1.1.2, the blood vessels have three layers composed of different cell types. Fibroblast and connective tissue form the adventitia, and the intima makes contact with circulating blood and inhibits thrombosis in the EC lining. The media, made up of SMCs and elastin, provides mechanical strength and integrity. It is ideal for vascular TEVGs to mimic these functional properties.

Studies have explored the impacts of vascular graft modifications to modulate ECM protein production during tissue formation. For example, endothelialization has been limited because adhered ECs detach from the surface when exposed to blood circulation, so poly(tetrafluoroethylene) (PTFE) was coated with fibronectin and RGD containing peptides to improve EC attachment [25]. It was concluded in this study that cell attachment and cell retention were significantly increased by modifying the surface [25]. Surface modifications of the biomaterial are important for the cellular component and overall are essential for the regenerating tissue.
1.3 Biomaterial Choice and Scaffold Fabrication

Properties of the scaffold are guided by the biomaterial used and fabrication process. Biodegradable and biocompatible properties for a tissue engineered scaffold can be impacted by the biomaterial composition and processing techniques used. Biomaterials relevant for tissue engineering are often categorized into the following three groups: ceramics, natural, and synthetic macromolecules. However, here we will only discuss the macromolecule approaches that have been tested for vascular tissue engineering applications.

1.3.1 Natural Materials

Cellular repopulation and tissue remodeling can be aided by many naturally derived materials [11,26]. Collagen and elastin are structural proteins produced in the body, and they are used in tissue engineering systems because they induce cell attachment and cell signaling through bound cells (e.g. integrin signaling) [26,27]. Cells can also enzymatically degrade most naturally-derived materials, which impacts the matrix remodeling and cell phenotype [28]. For example, other natural scaffolds (from decellularized blood vessels) have been used because they provide growth factors, proteoglycans, glycosaminoglycans, and structural proteins that benefit the cells. But, these decellularized scaffolds for vascular grafts do not have enough porosity to support cell seeding in in vitro conditions and ultimately lack long-term mechanical properties [29].
1.3.2 Synthetic Materials

Non-degradable Synthetic polymers are often used for vascular applications because they have many benefits including high tensile strength [26,30]. Examples of non-degradable polymer materials are ePTFE and Dacron (polyethylene terephthalate (PET)), and both have been used as larger diameter vessels (6-10 millimeters) for larger peripheral arteries. However, it is known that extended inflammatory responses prevent the progression of a functional endothelium in the lumen of the vessel. This results in atherosclerosis in the native vessel [31] and in synthetic ePTFE grafts [32]. It is concluded that these non-degradable synthetic materials, when used in small diameter vessels, lose functionality as a result of occlusion and intimal hyperplasia [13–15]. Therefore, it is imperative to discuss a more degradable material for scaffolds used as small diameter vessels.

Biodegradable polymers possess an ability to breakdown over time and the byproducts are naturally engulfed plus they can be made non-toxic to the body. Poly(ortho esters) are a family of synthetic degradable polymers commonly used for medical devices [26,30]. There are also many others that have the ability to degrade and remodel after implanting into a graft site. Some examples are polyurethanes, poly(glycolide) (PGA), poly(lactic acid) (PLA), poly(ε-caprolactone) (PCL), and highly cross-linked polyesters such as poly(glycerol sebacate) (PGS) [26,30,33,34]. These materials can also be formulated to undergo distinctive degradation processes (e.g. bulk diffusion), which are sought after for drug delivery in tissue engineering.
applications. **Figure 1.3** demonstrates several important scaffold properties that influence drug release in tissue engineered systems [35].

---

**Figure 1.3.** Illustrates a few important properties of scaffolds that impact drug release. These include (A) swelling of low density hydrogels, (B) bulk degrading polyesters, (C) surface erosion in polymers such as polyanhydrides in an aqueous environment, and (D) covalent conjugation of proteins. The resulting impact on the release profiles include very fast, burst, controlled, and extended or long term availability, respectively, for a representative pharmaceutical agent. The dashed line represents the initial boundary of the material. Reprinted from an open access journal [35].
This illustrates that scaffold properties are impacted by the polymer material used, and as a result, the release rate varies. Details of drug release will be discussed in this dissertation; however, it is also reviewed extensively in another paper [35]. Importantly, it must be noted that the method of processing for the scaffold will also effect the release of drug within the delivery system.

1.3.3 Scaffold Fabrication Techniques

A major criteria of tissue engineering scaffolds is proper porosity allowing the infiltration of cells in addition to transport of nutrients and gases [36]. The scaffold should have a porous network that permits diffusion of nutrients; this will assist in the development of a viable and functional graft [37]. There are many different processing techniques that produce scaffolds with a porous structure. They are gas foaming, phase separation, fiber bonding, extrusion, emulsion freeze drying, solvent/casting, particulate leaching, rapid prototyping, and electrospinning [30]. The native ECM is a well-organized fibrous structure, and a fibrous structure is ideal for TEVGs. Electrospinning is a common method to fabricate a fibrous scaffold with properties similar to the native vessel; therefore, this technique will be the focus of this study.
1.3.3.1 Electrospinning

Electrospinning is a processing method that generates fibers with diameters ranging from micrometers to nanometers, specifically 3 nm to greater than 5µm [38]. The technique is inexpensive, and the simplistic nature of the process has made electrospinning a popular method for fabricating fibrous scaffolds. Electrospinning works by the use of an electrical charge to generate fibers from polymer solutions or melts. The system consists of three main components: a high voltage power source, a syringe pump, and a rotating metal collector (Figure 1.4). During the process, the polymer solution is pumped through a syringe, and held at the needle tip by surface tension. A high voltage is applied to the needle, which induces a charge within the polymer solution, while the rotator drum is grounded. The electric field overrides the surface tension of the solution, and results in a charge repulsion. Before reaching the rotator drum, most of the solvent evaporates, leaving small fibers upon the rotator drum. These fibers are collected on the target and form scaffolds.
There are several parameters that impact the electrospun fibers that can be categorized as controlled and solution variables. The controlled parameters include the distance between the needle’s tip and collector, the electric field strength, flow rate, and the geometry and composition of the collector. Solution properties include conductivity, dielectric constant, dipole moment, molecular weight of the polymer, surface tension, and viscosity. These variables affect the individual electrospun fiber size and morphology. For example, the viscosity of the solution is controlled by altering the polymer concentration, and it has been found that this parameter has the greatest impact on fiber size and morphology when electrospinning polymeric fibers. Studies have electrospun poly (lactic-co-glycolic acid) (PLGA) [39], poly (DL-lactic
acid) (PDLA) [40], poly (L-lactic-acid) (PLLA) [41], polystyrene [42], gelatin [43], and dextran [44].

1.4 Drug Delivery through Tissue Engineered Systems

Healthcare has benefited from the development of new drug delivery systems. The drug delivery system can be incorporated within the design and fabrication of biocompatible scaffolds, can be incorporated within, specifically by controlling the spatiotemporal release of biological factors to direct cell behaviors and gradually leading to regeneration of tissue [45]. However, the ability to achieve localized and controlled delivery of therapeutic molecules in a three dimensional (3D) scaffold is another important parameter for tissue regeneration and growth [45]. For example, the controlled release of angiogenic factors, including fibroblast growth factors or vascular endothelial growth factors, can improve vascularization needed for maintaining continuous blood supply to developing tissues.

Vascular tissue engineering translates the vast knowledge of vascular biology to develop new therapy options for many clinical disorders [46]. Blood vessels act as conduits to transport nutrients and oxygen to and waste products away from tissue and organ systems. In addition, vessels must maintain mechanical integrity such as withstanding a wide range of pressures and shear stresses, regulating blood flow and permeability, and repelling thromboses under basal conditions [46]. These vessels must also consider the immunological response of the vascular tissue engineered system. The inflammatory response in TEVGs is often modulated by the material
properties and through the controlled release of pharmacological agents (to be discussed in next section). A detailed review of these molecules has been conducted in our review article [35].


1.5 Bioactive Molecules for Cardiovascular Therapy

Several growth factors, small-molecule drugs, and other bioactive molecules have been released from tissue engineered scaffolds, but relatively few have been incorporated for the generation of vascular grafts for artery replacement [35]. Most of the molecules released from vascular scaffolds have been anti-inflammatory or anti-oxidant compounds. Table 1 lists bioactive molecules that have been released from vascular scaffolds [35]. Some include fibroblast growth factor-1 (FGF-1) that demonstrated an anticoagulant response [47–49], anti-CD34 antibody that increased endothelialization [50–52], ascorbic acid or citric acid that maintained viability in high ROS environments [53,54], and heparin that promoted endothelialization and smooth muscle cell (SMC) proliferation [47,55,56]. Gasotransmitters, such as nitric oxide [53,57–59] and carbon dioxide [60–62] have also been released from vascular scaffolds. These elicit inflammatory responses included induced vasodilation [53,59,63], inhibited platelet aggregation [53,57–59], and impeded expression of
pro-inflammatory cytokines [60–62]. Most tissue engineered strategies focus on modulating and quickly resolving, but not avoiding an inflammatory response because tissue deposition is required in early stages. In later stages, tissue deposition should decrease, and cells should become more like those in the native environment [35]. If this does not happen, foreign body giant cell formation can occur [64]. Therefore, the overall goal is that the inflammatory response initiates remodeling, but subsides quickly to allow the presence of vascular cells demonstrating a phenotype similar to those in a healthy vessel in addition to extracellular matrix maturation [35].

It is essential to also understand the tissue and cells of the diseased condition within the native artery to determine the inflammatory response. As previously discussed in Section 1.2.3, cells are an important component of the tissue engineering strategy, but it is imperative to also understand the native tissue. There are several types of cells found within the layers of an artery [65], and they play an important role in the tissue. In a healthy intimal layer, there are confluent endothelial cells that prevent initiation of the coagulation cascade, and give a paracrine signal to the SMCs within the medial layer that help maintain the quiescent SMC phenotype [35]. But ECs in diseased conditions do not demonstrate proper function. For example, these ECs do not have cell-cell signaling through gap junctions, in particular connexins 37, 40 and 43 [66,67] and this can result in hyperproliferation of SMCs. SMCs are found in several layers in the middle of the vessel, and they contribute to vascular tone, as well as activation and migration of SMCs into the intima that is an important step in
atherosclerotic lesion formation [68]. Other cells, including fibroblast and pericytes, are found in the adventitial layer, and it is important to note that all of these cells in the diseased artery release pro-inflammatory cytokines that would impact a graft and its viability [69,70]. With these reasons, it is vital to deliver bioactive compounds that have shown promise for treating atherosclerosis in vascular tissue engineered vascular grafts. More specifically a gasotransmitter such as carbon monoxide.
Table 1. Bioactive molecules released from vascular scaffolds

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Response</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antioxidants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid/ Citric acid</td>
<td>Maintained cellular viability in high ROS environment</td>
<td>[53,54]</td>
</tr>
<tr>
<td>Penta-galloyl glucose</td>
<td>Reduced degradation of the scaffold by matrix metalloproteases</td>
<td>[70], [71], [72], [73]</td>
</tr>
<tr>
<td><strong>Gasotransmitters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>Inhibiting the expression of pro-inflammatory cytokines; promoting interaction with local cell target</td>
<td>[60–62]</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>Mediates vasodilation and inhibit platelet aggregation.</td>
<td>[53,57–59,71]</td>
</tr>
<tr>
<td>S-nitrosothiols</td>
<td>Induced vasodilation</td>
<td>[53,59,63]</td>
</tr>
<tr>
<td><strong>Glycosaminoglycans</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>Promoted endothelialization and SMC proliferation;</td>
<td>[47,55,56]</td>
</tr>
<tr>
<td><strong>Growth factors &amp; other Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroblast growth factor-1</td>
<td>Anticoagulant; vessel sprouting mediates interaction with ECM;</td>
<td>[47–49]</td>
</tr>
<tr>
<td>Anti-CD34 antibody</td>
<td>Increased endothelial cell</td>
<td>[50,51]</td>
</tr>
<tr>
<td>Transforming growth factor beta 1 (TGF-β1)</td>
<td>Promoted contractile protein expression by SMCs; reduced ring thickness; and promoted TEVG remodeling</td>
<td>[52]</td>
</tr>
</tbody>
</table>

1.6 Benefits of Carbon Monoxide (CO)

Gasotransmitters are inorganic gases that have a unique role in cellular signaling in biological pathways. These molecules are naturally occurring vasodilators, and they have shown anti-inflammatory effects at appropriate doses [72,73]. Carbon monoxide (CO) is produced endogenously in the body when heme oxygenase (HO) enzyme degrades heme molecules [74]. CO is most commonly known because, at high doses, it is pro-inflammatory or even fatal. However, at appropriate doses, CO has anti-inflammatory properties [75]. HO-1 has been shown to provide oxidative stress protection by itself [76]. However, CO has also independently been shown to provide anti-inflammatory properties. For example, it has been shown that CO acts by inhibiting the expression of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin-1beta (IL-1β), and macrophage inflammatory protein-1 beta (MIP-1β), as well as by increasing the production of the anti-inflammatory cytokine IL-10 [60]. In addition, inhaled CO therapy has been investigated in pre-clinical models and is currently in a phase II clinical trial for treating pulmonary fibrosis in adult patients [77]. In order to successfully develop a TEVG with incorporated CO delivery, it is required to better define CO dose levels and identify CO signaling pathways.

1.6.1 Cell signaling: A result of carbon monoxide

CO has been shown to occur through binding to other heme-containing compounds within the cell, and early work focused on CO binding to sGC that leads to increased cGMP production. This is a very beneficial pathway, and it is also the best characterized pathway. It has been shown to lead to impacts such as vasodilation, increased thrombolysis, and decreased SMC proliferation [78–80]. However, other heme-containing targets for CO, and the corresponding pathways have continued to be identified. **Figure 1.5** illustrates some of those pathways that explain different impacts of CO on different cell types [35]. For example, CO can promote EC proliferation, but reduce SMC proliferation [80,81]. These studies indicate the potential impacts of CO towards prevention of intimal hyperplasia and vessel stenosis. The suppression of SMC proliferation has been linked to CO binding to either sGC or NOS, and the signaling proceeds through a cGMP-dependent pathway [82–84]. This has also been shown to involve transcription factors (e.g., E2F) [80] and proteins that regulate the cell cycle, including increased levels of the cyclin-dependent kinase inhibitor p21[85]. But, when CO binds with NOS, it involves Ras homolog gene family member A (RhoA) and Akt, and it promotes EC proliferation [86]. This is one of the pathways that demonstrates an interaction between both the
gasotransmitters CO and NO. In addition, CO has been shown to provide anti-apoptotic effects on endothelial cells through a p38 mitogen activated kinase pathway (MAPK) that includes upregulation of anti-apoptotic genes [e.g., inhibitors of apoptosis 2 (IAP2)] [87]. Interestingly, CO may also provide dose-specific modulation of cellular apoptosis through mitochondrial swelling. Therefore, it is important to investigate CO-dose and detection of CO uptake into the cell.
Figure 1.5 Carbon monoxide (CO) Pathways and heme-binding targets. (A) cGMP-dependent pathway that leads to suppression of SMC and decrease thrombosis. (B) CO interacts with RhoA and Akt to promote EC proliferation. (C) A p38 mitogen activated pathway that exhibits anti-inflammatory effects. Reprinted from an open access journal [35].
1.6.2 Carbon Monoxide Releasing Molecules (CORMs)

Drug delivery from vascular tissue engineering systems is being investigated as an alternative graft because greater than 30% of patients exhibit multiple plaques and do not have viable veins for grafting [1,88,89]. But studies have shown this treatment intervention also has drawbacks including thrombosis, intimal hyperplasia, and limited endothelium function [90,91]. Because of CO’s inherent toxic nature at high doses, it is important to avoid toxic effects and side effects to surrounding tissues when delivering this drug molecule. Therefore, it is critical to locally control the dose of CO through carbon monoxide releasing molecules (CORMs).

The recently synthesized CORMs provide a promising delivery approach to reduce the safety concerns of systemic, inhaled CO gas. The potential to control the release of CO to a specific target is the major advantage of CORMs over gaseous CO as a therapeutic agent. CORMs must meet specific requirements such as chemical stability, solubility in aqueous media, low toxicity of both CORMs and their degradation products, and a triggered release mechanism [92,93]. CORMs can be categorized based on either their chemical structure or the method of release. Several other review articles have been published on general approaches of triggered CO release based on acidification, thermal release, ligand exchange (CORM-2), ligand substitution (CORM-3), enzymes (ET-CORM) and light (photo-CORM) [93,94]. Various other CORMs have been developed, and have been described in several review articles [95,96]. The classes of these CO-releasing molecules can be categorized as aldehydes, boroncarboxylates, metal carbonyl complexes,
organometallic compounds, oxalates, and silacarboxylates [97]. However, the majority of these compounds are metal carbynols that quickly release CO in the presence of water, many of which contain heavy metals. There is also a boron carbonate compound that does not contain heavy metals and releases CO at a slower rate. However, CORMs that release CO in response to light are being investigated to allow for a more controlled release.

These CORMs have the ability to transport and deliver CO in biological systems. CORMs are alternatives to CO gas and the limitations of inhaled CO including toxic or lethal at higher levels and uncontrolled delivery. Others have used CORMs that release CO by hydrolysis under physiological conditions, but in in vivo scenarios, the ability to activate these CORMS is reduced because of a short half-life. This concern can be overcome by the use of CORMs that release CO in a precise spatial and temporal controlled manner by photo-irradiation.

1.6.2.1 Photo-Induced CORMs

Photochemical systems can be used to control the release of biologically active species and convert a non-conjugated species to a conjugated species using visible light. This photochemical system is related to the photo-retro-Diels-Alder (PrDA) reaction. It is a novel type of organic carbon monoxide releasing molecule (photo-CORM) developed by our collaborator Dr. Yi Liao. The photoCORM releases CO under irradiation with visible light and simultaneously produces a fluorophore that allows one to detect the delivery and the extent of CO release.
In aqueous media, diketone-based CORMs undergo hydration and form hydrates that are not photoactive. Thus, a hydrophobic carrier is required to protect CORMs from hydration and activate these CORMs to release CO under physiological conditions. In a previous study, Pluronic F127 micelles were used as the hydrophobic carrier, which have been widely used as drug carriers. However, these micelles can be washed away, and they do not result in complete CO yield [98]. Fibrous scaffolds, such as what we used in this dissertation, have the ability to overcome these limitations.

Specifically, there were three unsaturated cyclic α-diketone (DK) based photo-organic CORM compounds that we used for these proposed studies. The first CORM compound is 9,10-dihydro-9,10-ethanoanthracene-11,12-dione (DK1), the second CORM compound is a more hydrophobic material and it is known as 2,6-bis(octyloxy)-9,10-dihydro9,10-ethanoanthracene-11,12-dione (DK3), and the third CORM compound (DK4). These will be discussed in the next three sections and understanding the pharmacokinetics and cellular response to these materials is a focus of this dissertation.
1.6.2.1.1 DK1

DK1, the unsaturated cyclic α-diketone is synthesized by Dr. Yi Liaos’s Lab according to Scheme 2.1. The cyclic Diels-Alder adduct (compound C5-1) is formed by reacting anthracene with vinylene carbonate at a high temperature (150 °C). The resulting adduct is hydrolyzed using a 40% NaOH solution, to obtain the dihydroxy product (compound C5-2). The final DK1 compound is obtained in high yield via Swern oxidation of the dihydroxy compound [99].

Schema 1.0 Synthesis of DK1
**1.6.2.1.2 DK3**

DK3, a diketone molecule with two long -OC₈H₁₇ side chains is prepared as shown in **Scheme 2.2**. First, 2,6- dihydroxyanthracene-9,10-dione is subjected to etherification with 1-bromoocctane in the presence of potassium carbonate as the base. The resulting compound **C5-3**, is subjected to reduction using sodium borohydride to obtain an anthracene derivative with two long side chains (compound **C5-4**). Finally, DK3 is synthesized via three more steps, including a Diels-Alder reaction (compound **C5-5**), hydrolysis (compound **C5-6**), and Swern oxidation (compound DK3) similar to the synthesis of DK1 [99].

**Scheme 1.1 Synthesis of DK3 [102].**
1.6.2.1.3 DK4

DK4 is a diketone molecule that is synthesized as shown in Scheme 1.2. Firstly, Friedel-Crafts alkylation is used to react anthracene with t-butyl alcohol to obtain Compound 2. The cyclic Diels-Alder product is formed by reacting Compound 2 with vinylene carbonate at 150°C. Then it is hydrolyzed to make the diol Compound 4. Finally, DK4 is obtained via Swern oxidation of 4.

Schema 1.2 Synthetic procedures and characterization of products DK4
1.7 Experimental Plan

The overall goal of this dissertation was to generate a CORM-loaded electrospun scaffold that could be used as a small diameter vascular graft and to determine the impacts of CO on ECs response, which is necessary to develop a functional endothelium. This was achieved through two steps. The first step investigated the impacts of CO-loaded electrospun scaffolds on ECs through *in vitro* systems for cardiovascular applications. The second step determined mass transport of CO through diffusion-based computational modeling and drug delivery of gasotransmitters from tissue engineered scaffolds.

The first study was to determine the impacts of CO on endothelial cells to develop a functional endothelium (Chapter 2). To accomplish this, spin coated thin films and electrospun PCL scaffolds at varying concentrations of CORMs were fabricated, characterized, seeded with endothelial cells, cultured up to 14 days, and analyzed with biochemical assays and immunofluorescence stains. These tasks were performed with the help of several students including Natalie Shah, Kellen Maurus, and Kai Clarke.

The second task was to determine mass transport of CO through diffusion-based computational modeling and drug delivery of gasotransmitters from tissue engineered scaffolds (Chapter 3). This was completed through the design and development of a diffusion-based computational model. The model system was designed by Shawn Rottman as his master’s thesis project. Rubens Jourdain collaborated and finished additional simulations and modifications after Shawn
Rottmann graduated. My specific roles were to develop the model parameters by finding mass transport properties, to complete the experimental validation of the model and to write the manuscript. In this dissertation, I also furthered the discussion of the implications of the model for tissue engineering.

The conclusion of these studies and their contributions to the field of vascular tissue engineering, as well as three areas of further research, are described at the end of the document (Chapter 4). The results summarized there demonstrate the feasibility of using CORMs to impacts ECs and improve a tissue engineered strategy.

1.8 Materials within Appendices

A list of relevant articles and book chapter that summarize other projects that I helped develop is in Appendix A. In addition, Appendix B provides a brief overview of an ongoing project that investigates hemocompatibility of Collagen I-incorporated blended scaffolds as TEVGs.
2.1 Introduction

Occlusion of small vessels (< 4 mm) in coronary arteries results in reduced lower extremity function in approximately 20% of seniors [100]. The current gold standard for treatment is bypass grafting, but up to 30% of patients do not have viable supply of saphenous veins for grafting [1,101]. Therefore, tissue engineered vascular grafts (TEVGs) are being used an alternative bypass graft option, but TEVGs typically exhibit limited endothelial cell function [102]. Gasotransmitters such as carbon monoxide (CO) have the potential to improve vascular graft function because these are cell-signaling molecules produced naturally in the body with anti-inflammatory properties at appropriate concentrations [103]. Drug delivery of CO at appropriate doses from scaffolds provides local-controlled delivery of anti-inflammatory agents to the diseased area; however, it is imperative to understand the
impacts of our biomaterial properties on cellular behavior (e.g. cellular attachment and proliferation) and to determine effective CO dose levels.

The proper dose of CO that reflects beneficial responses of cells has yet to be determined because there are seemingly conflicting results in various tissues and studies. CO at endogenous levels exhibit anti-inflammatory properties and are required for vascular function. However, high levels of CO are toxic or pro-inflammatory, and there are still questions about the levels in between [104,105]. For example, the CO dose for systemic studies has typically been defined as either ppm in the inhaled gas or percent CO bound to a heme compound – carboxyhemoglobin (COHb) \textit{in vivo} in the blood or carboxymyoglobin (COMb) \textit{in vitro} tests. Finally, it is even more uncertain how COMb levels found with \textit{in vitro} studies relate to the results in clinical and in animal models. Therefore, understanding the impacts of CO on endothelial cells will provide a better understanding of cell response to CO released from CORMs through toxicity and biocompatibility for a functional graft.

Delivery of CO to the diseased area may improve the inflammation induced by hyperproliferation of cells and function of vascular endothelial cells. We previously determined the impacts of incorporating visible light activated CORMs into electrospun scaffolds [106]. We reported controlled CO release from scaffolds with visible-light activated carbon monoxide releasing molecules (CORMs). However, our results demonstrated the need to increase the maximum incubation time that permits CO release from organic CORM-loaded scaffolds [106].

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Cellular response and ultimately tissue regeneration is dependent on several important factors including implant biomaterials and surface properties. Biomaterials guide cell adhesion, migration and morphology, and contribute to cell survival [107]. We investigated the impacts of CO on ECs seeded on scaffolds and spin-coated thin films to better understand the two-dimensional (2D) and three-dimensional (3D) property effects. Seeding on 3D scaffolds gave insight into how cells will respond and behave in the native environment. In vitro studies generally illustrate favorable cell responses to charged, hydrophilic surfaces, corresponding to superior adsorption and bioactivity of adhesion proteins, but this doesn’t truly represent the in vivo environment with blood and interstitial fluid present. Further, implanted materials may need surface modifications to promote the optimal cell response. For example, coating with extracellular adhesion proteins and growth factors contribute to cell attachment, survival, growth, and differentiation. These properties are discussed in details in a review article [107].

In this study, we aimed both to extend the culture time to allow CO release by using a more hydrophobic CORM (DK3) and to determine the endothelial cell (EC) response to this material. In addition, we will advance our understanding of how the CORM (DK3) can be manipulated to influence primary EC viability, ROS products, and ROS levels. There was also a newly synthesized CORM material which overcomes challenges of electrospinning a waxy material or with DK3 that was tested. The toxicity and biocompatibility of this newly synthesized CORM (DK4) was tested when loaded within poly(ε-caprolactone) (PCL) thin films as well.
as nanoparticles to expand the use of CORMs within other drug delivery systems. Finally, the in vivo biocompatibility is also explored in a pilot study to determine patency, toxicity and viability.

2.2 Methods and Materials

2.2.1 Materials

All disposables, chemicals and biological supplies were purchased from Fisher Scientific (Pittsburgh, PA). Poly (ε-caprolactone) (inherent viscosity IV = 1.2 dl*g⁻¹) was purchased from Lactel Absorbable Polymers (Pelham, AL). Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC and PromoCell.

2.2.2 CORM Synthesis

The DK1, DK3, and DK4 compounds were synthesized as discussed in detail within Chapter 1. The preparation of the CORM compound, 9,10-dihydro-9,10-ethanoanthracene-11,12-dione (DK1), was performed in three steps. DK3 was prepared as a five-step synthesis including a Diels-Alder reaction, hydrolysis and Swern oxidation [99]. DK4 that was synthesized by Friedel-Crafts alkylation that replaced anthracene with t-butyl alcohol. The cyclic Diels-Alder intermediate was formed by reacting with vinylene carbonate at 150 °C. Then it was hydrolyzed, and obtained via Swern oxidation of the cyclic Diels-Alder intermediate.
2.2.3 Electrospinning and Spin Coating

Poly (ε-caprolactone) (PCL) was used to electrospin 0 and 2 % w/w CORM-incorporated scaffolds. SEM and Image Pro Software were used for material characterization.

PCL was also used to spin coat and test three different concentrations of DK4 solutions within the film, 1%, 0.1%, and 0% (w/w), along with a tissue culture polystyrene (TCPS) control. The DK4 loaded films were prepared with a solution concentration of 6% (w/w) DK4 and 12% PCL in hexafluoro-2-propanol in HFIP. The solutions were spin coated into thin films with consistent thickness of ~3-5 nm by adding 250 µL of solution [Program parameters = 1200 RPM, Acel +1200, Step 001/001, Time 30 seconds].

2.2.4 Scaffold Characterization

The electrospun scaffolds were sputter-coated with gold and then imaged in a JEOL 6380-LV SEM (Peabody, MA) with a working distance of 9 mm and operating at 5 kV. ImagePro Plus software (ImagePro® Media Cybernetics, Bethesda, MD) was used to determine the average fiber diameter from the SEM images.

2.2.5 Activation Profiles

We compared activation profiles of wet versus dry for all scaffold conditions. We determined how CORM-loaded scaffolds release CO in cell culture conditions and compared cell response to DK3 the more a hydrophobic material. Scaffolds were
activated with 470 nm light and CO release was tracked through its fluorescence signal. Samples were irradiated for 15 minute intervals up to 1 hour and relative fluorescence unit (RFU) was measured to determine maximum release.

2.2.6 Cell Culture

Primary HUVECs [ATCC CRL-1730 and PromoCell] were cultured in standard cell culture conditions [95% air, 5% carbon dioxide (CO₂), 37°C, and complete culture media (PromoCell Endothelial Cell Growth Medium MV with ECs growth supplement and 1% penicillin/streptomycin)] prior to seeding at densities described for each test.

2.2.6.1 CORM impacts on EC Mitochondrial Function

ECs (seeding density of 5,000 cells/cm²) were cultured in EC Complete Media for 3, 5 and 7 days to determine cell response and viability on pure PCL controls, CORM incorporated materials, and TCPS. ECs were also seeded on TCPS and incubated for 3 days to investigate impacts of the CORM on the mitochondrial function using Seahorse XF Cell Mito Stress Test to determine the Oxygen Consumption Rate (OCR) (n=10). We also obtained other important parameters including ATP production and Spare Respiratory Capacity. Details and replicate studies will be conducted by Mahyar Sameti.
2.2.7 Toxicity, Biocompatibility, and Viability Test of DK4

Scaffolds were sterilized with ethylene oxide and incubated in DMEM with 10 % fetal bovine serum. ECs were cultured in Endothelial Cell Basal Medium at standard culture conditions for 3, 5 and 7 days to determine cell response and viability with PCL control and CORM incorporated materials \((n=3)\). Live/Dead assays were performed on samples to investigate proliferation.

2.2.7.1 DK4 incorporated within PCL thin-films

We tested three concentrations of DK4/PCL solutions within the film, 1%, 0.1%, and 0% (w/w), as well as TCPS controls \((n=3)\). All samples were ethylene oxide (EtO) sterilized for biocompatibility testing. Samples were either kept away from light or pre-activated with 470 nm light for 5 minutes prior to seeding cells to the surface (pre-activated condition). This allows for testing biocompatibility of both the CORM itself as well as the final molecule remaining after activation and CO release.

2.2.7.2 DK4-loaded PBCA Nanoparticles

Internalization of CORMs may be necessary for some therapeutic applications; therefore, we investigated the impacts of CO within nanoparticles. The toxicity of DK4 incorporated within poly(butyl cyanoacrylate) PBCA nanoparticles was determined by delivering a single dose of the DK4 nanoparticle solution to endothelial cells seeded on TCPS (seeding density of 7,500 cell/cm²). DK4-loaded nanoparticles were tested both for the non-activated and pre-activated conditions. We
prepared nanoparticles with 0, 0.5, 5, and 50 µg/mL of DK4 loaded nanoparticles (DK4+NP) in sterile EC complete growth media.

For spin coated thin film biocompatibility, cells were seeded on either TCPS (control) or spin coated films. Wells for spin coated films were pre-coated with poly(2-hydroxyethylmethacrylate) (poly-HEMA) to prevent cells from adhering to the wells and ensure measurement of cell activity only on thin films. For nanoparticle biocompatibility, ECs were cultured on TCPS and single doses of DK4+NP suspensions were added after 24 hours of seeding to allow for attachment of cells before treating.

2.2.7.3 Cell Study Analysis

For both types of surfaces, regular media changes were performed every 2 days until the end points. AlamarBlue Cell Viability Reagent (ThermoFisher Scientific) was used to quantitatively measure viability through cell metabolism at days 1 and 7. The DNA (Hoechst 33342; ThermoFisher Scientific) Assay was used to measure cell density at days 3 and 7. It is a cell-permeant nuclear counterstain that emits blue fluorescence when bound to dsDNA. To provide qualitative analysis and additional support of the results, we also conducted Live/Dead assays and captured confocal images of ECs after 11 days of culture (n=1).

2.2.8 In vivo Biocompatibility

We conducted a pilot study to investigate the impacts of the DK3 CORM in vivo. Electrospun conduits, 1 cm in length, were ethylene oxide sterilized, placed in
PTFE porous pouches 0.045" by 0.025" size diamond shaped pores (McMaster-Carr, Robbinsville, NJ) to prevent adhesions, and implanted intraperitoneally according to an IACUC-approved protocol using 200–225 g male Sprague-Dawley rats (Charles River, Wilmington, MA) using laparotomy. Enclosed conduits were removed after 4 weeks and grafted into the aorta of the same rat to test patency for 6 weeks. Conduits were transferred to freezing medium (DMEM + 10% FBS + 10% DMSO), and stored in liquid nitrogen until use.

2.2.9 Statistical Analysis

Results are presented as mean ± standard deviation for fiber diameter. Studies were replicated to ensure experimental reproducibility of trends unless otherwise noted. Significance was determined in JMP Pro 13 using one-way ANOVA with Tukey comparison (p<0.05).

2.3 Results

2.3.1 Scaffold Characterization and Activation

Electrospun PCL and PCL/ DK3 scaffolds were characterized, confirming fiber diameters of 1.48 ± 0.10 µm and 1.89 ± 0.05 µm, respectively (Fig. 2A & 2B). Activation of the PCL/CORM material was achieved by irradiating with 470 nm intensity light. Confocal light micrographs show scaffolds during pre-activation and fluorescence of fibers post-activation (Fig. 2C & 2D). This demonstrates that the CORMs are loaded within the electrospun fibers and it also illustrates that the CORMs within these fibers can be activated and tracked indirectly with fluorescence.
2.3.2 Release Profiles and *In vitro* Response

To determine the release profile of the more hydrophobic material, we activated the PCL/DK3 for 15 minute time intervals up to 1 hour. In addition, we tested different times for activation (e.g., 5 min on/5 min off) *(Figure 2.1A)*. We found that DK3 material presents a greater release than the DK1 material. Incubation impacts were verified by incubating scaffolds in complete media for multiple times, irradiating with 470 nm light, and taking fluorescence measurements after each time point *(Figure 2.1B)*. Release profiles of DK3-incorporated scaffolds demonstrate the ability for the scaffolds to be activated and release CO with light exposure after an extended culture period (71.4 and 79.1% of samples media soaked for 1 and 24 hours of incubation compared to the 3 min activation reading, respectively; *n = 3*).
For the DK3 material with activation, data showed that SMC cytoskeletal organization appeared to decrease but EC proliferation increased when CORM-loaded scaffolds were activated to release CO to the attached cells (Fig. 2.2A & 2.2B). This different response for the different cell types is consistent with results suggested elsewhere for other CORMs materials [105]. Finally, ECs showed an increase expression of vWF from 3 (Fig. 2.2C) to 7 days (Fig. 2.2D) additional cell characterization was performed. Figure 2.3 illustrates extended excitation spectra of PCL/DK3 scaffolds used to determine oxygen consumption rate (OCR) of EC seeded from the Cell MitoTest. This shows that early cell response at 3 days to released CO includes impacts on mitochondrial oxygen consumption rate and a 20% decrease in

**Figure 2.1** A) Relative fluorescence for all conditions, and B) Spectra comparison for DK3 CORM. Sig. from (*) DK3 Dry, (#) DK1 Dry, and ($) DK3 wet.
ATP production compared to control condition. We also observed a decrease in H$_2$O$_2$ with CORM activation.

**Figure 2.2** Phalloidin-labeled images show proliferation and elongation of ECs on PCL (A) and Activated PCL/CORM (B) scaffolds at day 7 of culture. IF images show increased vWF expression from 3 (C) to 7 (D) days on representative activated DK3 scaffolds.

**Figure 2.3** Oxygen Consumption Rate (OCR) analysis of control (i.e., ECs seeded without DK3) and experimental (ECs seeded with DK3 in well) conditions from Seahorse XF Cell Mito Test. [https://www.agilent.com/en/products/cell-analysis/seahorse-xf-consommables/kits-reagents-media/seahorse-xf-cell-mito-stress-test-kit](https://www.agilent.com/en/products/cell-analysis/seahorse-xf-consommables/kits-reagents-media/seahorse-xf-cell-mito-stress-test-kit)
A quantitative study of EC responses was also performed. Figure 2.4A shows a preliminary DNA analysis of ECs on electrospun scaffolds. The cell number on spincoated surfaces containing DK4 was assessed. Figure 2.4B shows Normalized AlamarBlue results (normalized to non-activated PCL) \((n=4)\). CORM activation also appeared to induce EC proliferation through 11 days of culture in the DK3 material. Surprisingly, this other test show higher cell attachment on DK surfaces, but does not show an impact of CORM activation on cell proliferation. Overall, these results demonstrate a variability in CO release from scaffolds that requires further investigation.

![Graph A](image1)

**Figure 2.4** A) DNA analysis of ECs seeded on PCL and PCL/DK3 meshes with the full and ½ area at culture day 11. B) Normalized (normalized to NA PCL) AlamarBlue Assay for HUVECs seeded on non-activated PCL, non-activated DK3 CORM, activated PCL and activated DK3 CORM \((n=3)\). (*) Significance from non-activated DK3.
Next, biocompatibility of the newly synthesized CORM material, DK4, was investigated to determine its impacts on ECs and overcome challenges working with DK3. The physical properties of this material is a powdery solid and it allows for accurate measuring instead of waxy DK3. DK4 was electrospun as described in Materials and Methods Section. To understand the impacts of DK4 on ECs, we seeded cells on spin coated thin-films and we analyzed cell function using AlamarBlue Assay (results are shown in Figure 2.5) and DNA Assay (shown in Figure 2.6). These normalized (i.e., to Day 1 PCL) biocompatibility results show that the DK4 material within spin coated thin films are not toxic to endothelial cells at the 0.1% and 1% loadings tested. It is imperative to note that it appears that there is less cell attachment and growth on all PCL films versus TCPS surfaces.
Figure 2.5 AlamarBlue results of HUVECs seed on TCPS, spin coated 6% PCL, spin coated 0.1% and 1% DK4 from Day 1 to Day 7 \((n=6)\). Normalized to non-activated PCL for both time points.
The biocompatibility results in films did not show toxicity, and next we investigated the possibilities of enclosing the DK4 within PBCA nanoparticles for delivery into the cells. We explored the impacts of DK4 within nanoparticles on EC function. Figure 2.7 demonstrates DNA analysis of ECs seeded on TCPS treated with varying concentrations of DK4 Nanoparticle solutions \((n=6)\). Live/Dead Confocal images are shown in Figures 2.8. There was no significant difference between conditions, even with a dose of nanoparticles up to 50 µg/mL where almost all of the imaged cells were alive at the 11 Day time point. Overall, the results indicate good biocompatibility for DK4/PBCA nanoparticles.
Figure 2.7 DNA analysis of HUVECS seeded on TCPS treated with varying concentrations of DK4 nanoparticle solutions ($n=6$). No significance was determined.
EC Cell Seeding Density on Endothelial Cells

Characterization of endothelial cell with biomaterial interaction is very important for the development of a tissue engineered vascular graft. Endothelial cell proliferation is highly dependent on cell-cell contacts; therefore, testing the effects of seeding density was conducted to determine the seeding density impacts on cell proliferation. We analyzed EC proliferation through AlamarBlue Assay. Figure 2.9 shows cell proliferation impacts due to seeding density. Results demonstrate a systematic increase in initial cell attachment with the increase in density from 0 to 100,000 total cells seeded. This indicates efficient cell seeding. All conditions proliferated from Day 1 to Day 3. Finally, these results also show a plateau of proliferation at Day 7 and Day 14 which is likely due high confluence and lifting of the cells. However, the significance in this study illustrates that the endothelial cells

Figure 2.8. Live/Dead confocal images of endothelial cells with varying dose at A) no drug delivery of 0 μg/mL; B) low dose of 0.5 μg/mL and C) at high concentration of 50 μg/mL for DK4 nanoparticle solution at day 11 (n=1).
used in our projects will grow with proper culture properties including cell density with an appropriate material for them to attach to. Our biomaterial will have to be optimized with sufficient ECM proteins to promote proliferation for a functional endothelium.

**Figure 2.9.** Alamar Blue analysis of HUVECs proliferation with density comparison on TCPS analyzed at Day 1, 3, 7 and 14 (n=3).
In vivo Pilot Study

A non-activated DK3 graft from the peritoneal cavity was grafted autologously into the abdominal aorta of the same rat for 6 weeks show. Position and patency of the graft was monitored over the grafting period using ultrasound images. Figure 2.10 shows in vivo biocompatibility of implanted graft with ultrasound. We demonstrate that non-activated control grafts remained patent. Finally, we conclude that the CORM-loaded scaffold can be grafted and support blood flow of small-diameter vessels via the pilot study.

Figure 2.10 PCL / 2 % CORM graft (A). Ultrasound color Doppler image of the non-activated old CORM condition (B) and non-activated new CORM (C).
2.4 Discussion

This study shows that DK3 increases CO release from scaffolds compared to DK1. We also confirm an extended maximum incubation time of at least 24 hours. With DK3, cytoskeletal organization appeared to decrease for SMCs but increase for ECs when CORM-loaded scaffolds were activated. This different response for the different cell types is consistent with results for other CORM materials. We have shown that both the CORM material itself and CORM activation is non-toxic and allows EC functional marker expression (e.g., vWF). Finally, we demonstrate that the CORM-loaded scaffold can be grafted and support blood flow in small-diameter vessels. Further cell studies are ongoing. We conclude that there is some endothelial cell attachment and proliferation when seeded on DK3 scaffolds up to day 14. But there is a need to investigate, modify and optimize the surface properties of our biomaterial for better cellular response.

We concluded that both DK3 and DK4 compound were not toxic to the endothelial cells and overall, this indicates good biocompatibility for our CORM loaded thin films, scaffolds, and nanoparticles. Live/Dead confocal images of ECs after 11 days of culture with high and low doses further supported this conclusion. However, there were concerns of cell attachment and survival on PCL surfaces, even without the addition of CORMs, that were improved with surface modification of soaking biomaterial in high FBS media prior to seeding. This helped cell attachment along with an increase in seeding density from 5,000 cells/cm² to 7,500 cells/cm².
Cell response varies with the changes in biomaterial surface. We tested CORMs within a 2D surface before adding the 3D complexity with the fibrous scaffold. The cell number on spin-coated surfaces containing DK4 was assessed and Figure 2.5 shows AlamarBlue results. These results suggest that cells are less metabolically active on spin-coated PCL than on TCPS. This was expected because TCPS is modified to promote protein adsorption and cell attachment [108,109]. The results also suggest that the cells are more metabolically active at day 7 on pre-activated spin-coated films with the higher concentration of DK4 (1%) than the other conditions. However, there were no significant differences. This suggested that the waste product embedded within the film may have a positive impact on the cells’ activity. It is also clear that cell number did not change with DK4 loading percentages, indicating that DK4 and its waste product after activation do not show signs of toxicity to HUVECs when contained within a polymer scaffold.

ECs were cultured on TCPS with different doses of nanoparticles loaded with DK4 to test the impact of more direct interaction with the cells. Figure 2.7 demonstrates DNA analysis of ECs seeded on TCPS treated with varying concentrations of DK4 nanoparticle solutions. Like with the DK4 contained within the films, there was no significant differences between conditions, even with a dose of nanoparticles up to 50 µg/mL. This is a concentration that has been shown to be toxic for particular types of nanoparticles, but nanoparticles in other studies (e.g., slightly different iron oxide nanoparticles) were shown to not be toxic at the same levels [110–112]. Overall, this indicates good biocompatibility for our CORM loaded
nanoparticles. Live/Dead confocal images of ECs after 11 days of culture with high and low doses further supported this conclusion (Figure 2.8). Overall, we conclude that the DK4+NP solution is biocompatible for HUVECs at normal doses.

In addition, in vivo pilot study results demonstrate patency, viability and cell infiltration through the DK3 incorporated-graft wall when conduits are implanted into the aorta of rats. Von Willebrand Factor of non-activated samples illustrates that an endothelial cell layer was formed in the lumen. These series of toxicity and biocompatibility studies lead into the determination of appropriate CO-dose levels for a tissue engineered vascular graft.

2.5 Conclusion

This study suggests that the more hydrophobic CORM increases CO release from scaffolds. We also confirm an extended incubation time of 24 hours. Finally, we have shown that both the CORM material itself and CORM activation or its byproduct are not toxic to ECs and allow expression of EC functional markers (e.g., vWF). In addition, we demonstrate that the CORM-loaded scaffold can be grafted and support blood flow of small-diameter vessels via the initial pilot biocompatibility study test. The variation in results of biocompatibility results and the understanding the differences in CO from in vitro to in vivo studies warrants investigation with computational modeling of CO transport.
3.0 Chapter 3: Simulation of drug delivery from tissue engineered electrospun-scaffolds by computational diffusion-based modelling

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3.1 Introduction

Interest in drug delivery of gasotransmitters including nitric oxide (NO) and carbon monoxide (CO) for biomedical applications has grown in recent decades because of their important roles in health and disease. Both NO and CO are naturally occurring vasodilators that have been shown to demonstrate anti-inflammatory effects at appropriate doses [113,114]. Neonatal patients with pulmonary hypertension have been treated with inhaled NO [115,116], and a recently completed phase II clinical trial also used inhaled CO for treating pulmonary fibrosis in adult patients [117]. In addition, CO delivered from carbon monoxide releasing molecule
(CORM)-loaded scaffolds can provide a local-controlled dose of cell-signaling molecules to a diseased area that can improve endothelialization in tissue engineered vascular grafts. Our previous studies included the incorporation of CO releasing molecules (CORMs) into electrospun scaffolds and determining the impacts of CO on endothelial cells, where we found that there is a need to better understand CO dose available to the cells.

CO can exhibit cell specific impacts such as anti-inflammatory response and CO is required for vascular function, yet high levels are toxic or pro-inflammatory, and levels in between demonstrate seemingly conflicting results in various studies and tissues [35]. The major challenge of gasotransmitters delivered for therapeutic treatment is the interpretation of defining low and high doses of CO because these are gaseous molecules with low solubility and they quickly diffuse within the biological system, making it hard to control dose. The effects on the cellular response from the delivery of bioactive compounds (e.g., gasotransmitters) are highly dependent on the local concentration of the species. The endogenous levels described by the Center for Disease Control (CDC) are similar to what a body would be exposed to in normal ambient air. However, the higher values used in other therapeutic studies suggest a potential benefit of supplemental CO at appropriate doses. It has been demonstrated that, at low to moderate levels, there are beneficial cellular responses and immune function [118], while higher concentrations may display oxidative stress that can be harmful to cells [119]. Further, it is still uncertain how COMb levels found in vitro culture conditions would translate to delivery within
in vivo environments such as animal and clinical models. Understanding the concentration of the bioactive compound available to the cell (at the microfiber scale) is imperative for our drug delivery system. Therefore, the use of computational and mathematical modeling of fluid and mass transport is necessary to better understand CO dose and delivery for successful treatment to the diseased area.

The complexity of CO delivery and tissue engineered scaffold parameters limit what we can detect experimentally. For instance, experimentally, we can track CO indirectly through fluorescence detection after CORM activation of certain CORMs, and we can confirm the presence of CO after binding to myoglobin. However, neither of these techniques actually tell us the amount of CO delivered into the cells. This is a crucial limitation with real-time experimental analysis. In addition, for CO and many other drug delivery systems the concentrations within microfibrous scaffolds cannot be measured. Finally, there are many parameters on the microfiber level, such as fiber diameter, orientation or porosity that can be controlled in a scaffold and little is known about how they impact drug delivery.

While the basic mechanisms of drug release are known (e.g. bulk diffusion, burst release, and erosion-controlled), understanding the concentration of drug available to cells in fibrous scaffolds requires development of a new model. Modeling the release and transport of a drug from a fiber scaffold is a very complex task because it requires simulating multiple events simultaneously. There are several requirements that the model must incorporate to precisely predict release from fibers. These include providing the ability to have profiled releases from the fiber or release
capsule structures, transporting the material through the transport medium, account for the loss due to cellular uptake and the loss to the surrounding vasculature, and allowing for the spatial resolution of the system to account for the detailed effects of the fiber structure (e.g., orientation, diameter, or volume fraction). For CO release from scaffolds, bulk diffusion is expected. The way that a drug is incorporated within the scaffold, and the composition of the scaffold, itself have important impacts on the release mechanism and on the success of a delivery strategy [35]. There have been simple diffusion empirical models designed in the past, but these models had limitations for systems with fast release. For example, Lewis et al. developed a simple one-dimensional model to investigate the interactions between the spatial and temporal oxygen profiles and cell distributions within a cartilaginous tissue construct [120]. The generalized assumptions for these models work well for most applications; however, they do not account for detailed spatial resolution information needed to understand the effect of fiber scaffold structure. It is necessary to model on the microfiber scale to better understand the boundary interactions and how they impact our drug delivery system [121].

In this study, we developed a model that could account for the effects of the fiber structure on the release and transport of CO to the cells and investigated the impacts of CO diffusion. The model is a two-part theoretical model designed to simulate diffusion of CO and other traditional synthetic drugs from engineered fibrous scaffolds, and determine the temporal concentration of drug available to cells. Therefore, we first investigated and validated a fiber structure generator and release
into the surrounding vasculature. We also explore other boundaries, such as release profiles to determine what percent of drug actually yields into the cells, to offer a better understanding of our drug delivery system for tissue engineered cardiovascular applications.

3.2 Materials and Methods

3.2.1 Materials

All chemical, biological and disposable supplies were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise specified. Poly(ε-caprolactone) (PCL), with an inherent viscosity (IV) 1.2 dL/g in chloroform (CHCl₃), was purchased from Lactel Absorbable Polymers (Birmingham, AL). All fluorescent molecules including fluorescein isothiocyanate isomer I (FITC) were purchased from Sigma-Aldrich (St. Louis, MO).

3.2.2 Experimental methods

For experimental validation of the model, PCL scaffolds were electrospun with incorporated FITC or carbon monoxide releasing molecules (CORMs), and their respective release profiles determined as described below.
3.2.2.1 Synthesis of CORMs

The CORM material used in this study, DK3, is an aliphatic chain modified diketone and was prepared by a 5-step synthesis shown in Figure 3.0 [101]. Anthracene derivatives were reacted with vinyl carbonate to form cyclic Diels-Alder adducts [98]. The Diels-Alder adducts were hydrolyzed in NaOH solution to form the dihydroxy compounds, which were oxidized to diketones via a Swern oxidation reaction. DK3 is also a photo-activatable compound that is activated by 470 nm light.

![Figure 3.0 Schema of DK3 A) synthesis and B) activation with light](image)

Figure 3.0 Schema of DK3 A) synthesis and B) activation with light [101].
3.2.2.2 Electrospinning

To assess CO release rate, electrospun scaffolds were prepared from PCL with and without 2% (w/w) of the CORM incorporated. Solutions were prepared at 12% (w/v) in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), and then electrospun onto a stainless steel cylindrical drum to produce fibrous sheets. Electrospinning was performed with a 22 gauge needle using a 15 kV potential, a throw distance of 15 cm, and a syringe flow rate of 0.8 mL/h. Separately, electrospun PCL scaffolds were prepared with incorporated FITC for validating the transport model. A 14 % (w/v) solution in HFIP was used to keep the average fiber diameter similar to the pure PCL control. A consistent thickness of the scaffold and random orientation of the fibers was achieved using controllable lateral movement and slow rotation (<100 rpm).

3.2.3 Scaffold Characterization

Scanning Electron Microscopy (SEM) and Image Pro Software were used for material characterization and parameters for the model. The electrospun scaffolds were sputter-coated with gold and then imaged in a JEOL 6380-LV SEM (Peabody, MA) with a working distance of 9 mm and operating at 5 kV. ImagePro Plus software (ImagePro® Media Cybernetics, Bethesda, MD) was used to determine the average fiber diameter and degree of orientation from the SEM images. The degree of orientation was characterized using angular standard deviation [122]. Scaffold thickness was measured from brightfield images of the cross-section of the scaffold.
taken with a ZEISS Axio Observer A.1 microscope (Carl Zeiss Microscopy, Thornwood, NY).

3.2.4 Release Profiles

To determine the release profile of a surrogate drug, FITC from electrospun scaffolds, samples of all conditions were cut in equal dimensions (~11 x 11 mm, L x W) and weights. Single pieces of dry and wetted samples ($n = 6$ each) were placed in 1 mL of phosphate buffer saline (PBS) and incubated at 37 °C for 0, 15, 30, 60, 360, and 720 minutes, as well as every day up to 7 days of incubation. At each time point, the PBS was removed and replaced. Solutions were removed at each time period and saved for analysis. Fluorescence was analyzed in a Molecular Devices SpectraMax microplate reader (Sunnyvale, CA) with excitation at 490 nm and emission at 525 nm.

To determine CORM activation and CO release, scaffolds were activated and fluorescence of the electrospun scaffold was measured to indirectly track CO release [123]. Small pieces with equal dimensions ($7.8 \pm 1.0$ by $6.8 \pm 1.1$ mm, L by W) and weights were cut and incubated in saline prior to activation with a 470 nm LED light. Activation was performed at intervals up to 3 min to determine the initial release profile for CO. Fluorescence was measured with excitation at 320 nm and emission at 490 nm using the SpectraMax microplate reader at each time point. The same sample was used for all of the time points in the activation study ($n = 3$ each).

3.3 Computational Methods

3.3.1 Formulation of model

A two-part model was created to model the delivery of therapeutic molecules from porous scaffolds with micrometer or nanometer diameter fibers, which is a commonly used approach for tissue engineering. The local dose available to cells for many drugs is hard to determine experimentally at this scale, and in the case of CO delivery from scaffolds, the time and spatial dependent profile is not currently possible to determine experimentally. The model is a three step diffusion-based model process shown in Figure 3.1. The first part of the model allows for a controllable 3-D representation of fibrous structures with cells and drug molecules that provide the physical structure for the model. The second part is a mass transport model that simulates the release of a therapeutic molecule from the fibrous structure, where the boundary conditions can be adjusted to simulate the situations in different cell culture or *in vivo* situations. Finally, the third step is the model output as release profiles or diffusion heat maps over time.
The focus of the model in this article is for drug molecules / scaffold systems that allow release through bulk diffusion. However, we also show that it can be easily extended to systems that include a burst release or that rely on an erosion-based release mechanism.

3.3.2 Fiber Structure Generator

Development of the Fiber Structure Generator (FSG) focused around developing a tool that could reasonably recreate a three-dimensional fiber scaffold based on easily obtainable parameters of the original tissue scaffold. The open source 3-D creation suite Blender release 2.79, and a custom Python code was used. Python code was developed to generate a fiber scaffold based on the properties of a fibrous scaffold. The basic fiber characterization parameters such as diameter, diameter

Figure 3.1 The three step diffusion-based model process.
standard deviation, angle of orientation, and angular standard deviation were used to create the organizational frame of the fiber structure, while the volume fraction determined the quantity of fiber objects that was required to create the structure. The fibers were developed from a randomly assigned fiber seed that is extended using a von Mises distribution function that considers the fiber orientation (Equation 1), with angle of orientation ($\mu$), angular standard deviation ($\sigma$), and the concentration parameter term ($\kappa$).

$$k = \frac{1}{\sigma^2}$$ \hspace{1cm} \text{Eq. 1}

After the fiber scaffold is created, elipsoidal cells are embedded within the scaffold and attached to the fibers. After the 3-D scaffold representation was created, it was voxelized over the whole domain since the 3-D rendering is a continuous system that is not able to be directly fed into the mass diffusion model. Each voxel was only assigned one object classification type. If a voxel happened to have overlapping objects the assignment priority is: Cell > Release > Fiber. This priority removed the intersection of fibers running through the cells and also preserved the drug capsule space in a voxel. The output from the voxel data was written to an ASCII file using comma-separated delimiters.
3.3.3 Fiber Diffusion Transport Model

The fiber diffusion transport model was created using Fortran 90 code to move a therapeutic molecule from the fiber scaffold or release capsule and transport it through interstitial fluid to the cells or surrounding sinks. The selection of a diffusion-based model in this current study was based on the premise that in the case of tissue implantation, where tissue damage has occurred, the local area will have little to no convection through it. This is because revascularization does not typically occur until after three days. For the transport model, the change in concentration is tracked at the boundaries between voxels and at the outer boundaries (i.e., into the sinks). The rate at which transport occurs is dependent on the classification of the two adjoining voxels and in the context of this research is referred to as the boundary interaction type (Step 2 of Figure 3.1). These represent the range of transitions that can occur, and include Interstitial Fluid to Cell (ItC) that is representative of the cellular uptake across the cell membrane. The sinks can represent similar types of tissue outside the representative volume element (RVE) or different types of tissue, such as vasculature, which would provide different rates of diffusion in that direction. Transitions, such as Fiber to Interstitial Fluid (FtI), also can consider how the drug partitions between the two phases.
3.3.4 Governing equations – Diffusion equations

The model moves mass across these boundaries as a first order mass transport model. The change in concentration for one voxel is expressed as **Equation 2**:

\[ \frac{dc}{dt} = (\frac{\partial c}{\partial t})_{F_1} + (\frac{\partial c}{\partial t})_{F_2} + (\frac{\partial c}{\partial t})_{F_3} + (\frac{\partial c}{\partial t})_{F_4} + (\frac{\partial c}{\partial t})_{F_5} + (\frac{\partial c}{\partial t})_{F_6} \quad \text{Eq. 2} \]

where \( F_n \) represents the number of face directions for which transport into and outside of the voxel can occur across. The transport will be limited to only the six faces of the voxels for this model. Given that each face can represent a different boundary interaction type, it is possible that different transport equations define \( (\frac{\partial c}{\partial t})_{F_n} \) for each face. Some of the equation types used in this study are seen in **Table 3.1**. This includes Fick’s Law for bulk diffusion and the inclusion of a partition coefficient for some interfaces.
<table>
<thead>
<tr>
<th>Equation Type</th>
<th>Equation</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>( \frac{dc}{dt} = A )</td>
<td>( A = \text{Constant} ) (g/s·cm(^3))</td>
</tr>
<tr>
<td>Sigmoid Logistic</td>
<td>( \frac{dC}{dt} = D \frac{\partial^2 C}{\partial x^2} )</td>
<td>( A = \text{Constant} ) (g/s·cm(^3))</td>
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<tr>
<td></td>
<td>( D = \begin{cases} \frac{A}{1+\exp(-B)}, x &lt; 0 \ 0.0, C &lt; C_{Fn} \ 0.0, t &gt; B \end{cases} )</td>
<td>( t = \text{time (s)} ) ( B = \text{exposure time (s)} )</td>
</tr>
<tr>
<td>Sink Constant</td>
<td>( \frac{dc}{dt} = A_i ), where ( i = 1,# ) sinks</td>
<td>( A = \text{Constant} ) (g/s·cm(^3))</td>
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<tr>
<td>Fick’s Law</td>
<td>( \frac{dC}{dt} = D \frac{\partial^2 C}{\partial x^2} )</td>
<td>( D = \text{diffusion coefficient (cm}^2/\text{s}) )</td>
</tr>
<tr>
<td>Non-analytic Sigmoid</td>
<td>( \frac{dc}{dt} = \frac{A \cdot t}{1+t} )</td>
<td>( A = \text{Constant} ) (g/s·cm(^3))</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( t = \text{time (s)} )</td>
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<tr>
<td>Fick’s Law (permeability)</td>
<td>( \frac{dC}{dt} = HD \frac{\partial^2 C}{\partial x^2} )</td>
<td>( P = \text{permeability coefficient (cm/s)} )</td>
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<td></td>
<td>( HD = P \cdot d )</td>
<td>( d = \text{membrane thickness (nm)} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( H = \text{partitioning coefficient} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( D = \text{diffusion coefficient (cm}^2/\text{s}) )</td>
</tr>
</tbody>
</table>
A consumption term is also included for metabolism of the drug or other types of loses (e.g., CO binding to heme compounds within a cell). This decay term is shown in Equation 3 with the rate of consumption \( (R_{ci}) \) that shows the net loss of a drug in a voxel:

\[
\frac{dc}{dt} = \sum_{k=0}^{n} \left( \frac{\partial c}{\partial t} \right)_{F_{kn}} + \sum_{l=0}^{i} R_{ci} \tag{Eq. 3}
\]

For this project, the diffusion transport model uses \( i \) to represent the interstitial, fiber, and cellular phase. The \( R_{ci} \) term can be used to define any constant addition or loss term not accounted for by the transport, such as cellular consumption, chemical reactivity, and permanent binding.

The voxel structure created for this model allows us to simplify the solution to a discrete function of ordinate differential equations (ODEs). The Double-precision Livermore Solver for Ordinary Differential Equation (DLOSDDE) was incorporated into the model (Center for Applied Scientific Computing Lawrence Livermore National Laboratory Livermore, CA) to handle the large system of banded ODEs that are generated. The transport model equation for this project utilizes DLSODE to solve a stiff system with an internally generated banded Jacobian matrix. The upper and lower half bandwidth are equal to the number of rows in the voxel domain by the number of columns. Overall, the model architecture developed for the transport model allows for a large variety of different diffusion applications to be created and tested.
3.3.5 Scaffold Structure Templates

Different three-dimensional fibrous structures were created based upon the properties of electrospun scaffolds for all of the drug diffusion simulations. This included structures prepared from SEM images of electrospun meshes from literature sources to validate the ability of the model to recreate the physical structure size, shape, and dimension of experimental structures [125], as could be generated by electrospinning with a variety of materials. These same fibrous structures were also used in simulations to test the impact of different fiber scaffold parameters (e.g., fiber diameter) on CO mass transport. The fiber diameter, fiber orientation, and angle of orientation relative to the vertical axis of the images are described in Table 3.2. A fiber volume fraction of 30% is assumed for these structures. An additional condition (S3) with a higher volume fraction is also included to test the impact of this parameter. The intent of this scaffold is to fill in the gap between the characteristics for Scaffold 1 and Scaffold 2 with respect to fiber diameter and angular standard deviation. Finally, the parameters measured from the PCL meshes electrospun for this study were used in the simulation to validate the model’s release profiles. All tested scaffolds could realistically be created using standard electrospinning methods.

These scaffold geometries were used with values for transport rates for particular scaffold materials and delivered therapeutic molecule. Particular diffusion model parameters for the fast diffusing gas CO and a model small molecule, FITC,
are described in detail below. Other system tests were also tested, such as for albumin release, but these are not a focus of this article.

Table 3.2 — Characterization Parameters for PLGA Scaffold [124]

<table>
<thead>
<tr>
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<th>Parameter</th>
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<td>± 0.60</td>
<td>± 0.31</td>
<td>µm</td>
<td></td>
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<td>0.0</td>
<td>0.0</td>
<td>degrees</td>
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</tr>
<tr>
<td>Angular Standard Deviation (phi)</td>
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<td>0.0</td>
<td>0.0</td>
<td>degrees</td>
<td></td>
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<tr>
<td>Angle of Orientation (theta)</td>
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<td>0.0</td>
<td>0.0</td>
<td>degrees</td>
<td></td>
</tr>
<tr>
<td>Angular Standard Deviation (theta)</td>
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<td>52.5</td>
<td>10</td>
<td>degrees</td>
<td></td>
</tr>
<tr>
<td>Desired Volume Fraction</td>
<td>30</td>
<td>30</td>
<td>40</td>
<td>%</td>
<td></td>
</tr>
</tbody>
</table>
3.3.6 CO parameters

CO release from a scaffold is of particular interest to determine the impact of different scaffold parameters on drug delivery. The diffusivities and partition coefficients for CO were obtained from the literature and these are shown in Table 3.3 [124]. The diffusion parameters that involve transitions from fiber voxels are for transport of CO from a poly(lactic-co-glycolic acid) (PLGA) scaffold, which was the composition for the fiber scaffolds from the literature that we recreated in Section 3.3.5. Where values could not be obtained for carbon monoxide directly, surrogate species were selected based on similarities in parameters such as hydrophobicity, water and lipid solubility, other transport properties, and data availability. For example, in the fiber-to-interstitial reaction, no readily obtainable data for carbon monoxide was found. However, data for carbon dioxide (CO₂) was found, and while CO₂ is not directly comparable to CO, the ratio between the partitioning coefficients for two molecules was used to scale the rate to be more appropriate for CO [50]. For the model system, all of the edges or sinks were assumed to be to the same type of voxel (e.g., fiber-to-fiber) on the other side of the representative volume. The front sink had a constant concentration of zero that would be modeled as a vascular sink with convective flow of the drug out of the system. Finally, the CO was assumed to react or bind to heme containing compounds when within the cell.
### Table 3.3 — Carbon Monoxide MECH Definition [124]

<table>
<thead>
<tr>
<th>Boundary Interaction Type</th>
<th>Equation Type</th>
<th>Equation</th>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ItI</td>
<td>Fick’s Law</td>
<td>$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2}$</td>
<td>D (cm$^2$/s)</td>
<td>$3.0 \times 10^{-5}$[a]</td>
</tr>
<tr>
<td>ItC</td>
<td>Fick’s Law (permeability)</td>
<td>$\frac{\partial C}{\partial t} = \frac{HD \frac{\partial^2 C}{\partial x^2}}{HD = P \ast d}$</td>
<td>P (cm/s)</td>
<td>42 [b]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>d (nm)</td>
<td>4.0 [c]</td>
</tr>
<tr>
<td>ItS</td>
<td>(treat as ItI)</td>
<td>$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2}$</td>
<td>D (cm$^2$/s)</td>
<td>$3.0 \times 10^{-5}$[a]</td>
</tr>
<tr>
<td>FtI</td>
<td>Fick’s Law</td>
<td>$\frac{\partial C}{\partial t} = \frac{HD \frac{\partial^2 C}{\partial x^2}}{HD = P \ast d}$</td>
<td>P (cm/s)</td>
<td>42 [b]</td>
</tr>
<tr>
<td>FtF</td>
<td>Fick’s Law</td>
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<td>D (cm$^2$/s)</td>
<td>$1.0 \times 10^{-7}$[e]</td>
</tr>
<tr>
<td>FtC</td>
<td>(choose rate limiting for FtI or ItC)</td>
<td>$\frac{\partial C}{\partial t} = \frac{HD \frac{\partial^2 C}{\partial x^2}}{HD = P \ast d}$</td>
<td>P (cm/s)</td>
<td>42 [b]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>d (nm)</td>
<td>4.0 [c]</td>
</tr>
<tr>
<td>FtS</td>
<td>(treat as FtF)</td>
<td>$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2}$</td>
<td>D (cm$^2$/s)</td>
<td>$1.0 \times 10^{-7}$[e]</td>
</tr>
<tr>
<td>CtC</td>
<td>Fick’s Law (w/ chemistry)</td>
<td>$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} - (R_{C\alpha})$</td>
<td>D (cm$^2$/s)</td>
<td>$3.0 \times 10^{-5}$[a]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$R_{C\alpha}$ (g/s*cm$^3$)</td>
<td>$1.0 \times 10^{-6}$[f]</td>
</tr>
</tbody>
</table>

[a] Carbon monoxide in water (collapsed gas bubbles in water) $2.43 \times 10^{-5}$ cm$^2$/s at 30°C, while $3.62 \times 10^{-5}$ cm$^2$/s at 40°C [126].

[b] Oxygen permeability coefficient for the plasma membrane is about 42 cm/s [127]. O$_2$, NO, N$_2$, and CO behave quite similar in terms of water and lipid solubility, as well as partition coefficients; therefore, the O$_2$ permeability is being used as a surrogate for CO.

[c] An overall bilayer membrane thickness of 4 nm, with 3 nm being strongly hydrophobic and the rest being composed of the polar heads [128].

[d] The rate from [d] was scaled by the oil to water (H$_{ow} = 4.47$) partitioning coefficient and hydrophobicity. Since we are considering the opposite direction of travel (e.g., oil to water), the inverse for the partitioning coefficient was used multiplied times two, since PLA has a contact angle of 61° compared to 126° for polyethylene.

[e] CO$_2$ diffusivity is about $2.0 \times 10^{-7}$ cm$^2$/s for PLA and $1.0 \times 10^{-7}$ cm$^2$/s for PLGA [129]. CO$_2$ is being used as a surrogate for CO in fiber diffusivity.

[f] The $R_{C\alpha}$ is used as the rate of cellular consumption. The rate of CO consumption by the cell is currently unknown, but a moderate level of consumption is being applied to prevent build up in the cell, without significantly perturbing the system.
The initial tests assumed that the CO was all released instantaneously when a light was shone on the scaffold for activation. However, the CORM activation and CO release is a function of light intensity and duration.

3.3.7 FITC release parameters

The release of FITC from electrospun scaffolds was used for validation because it is not possible to experimentally assess CO release in real time. FITC release from PCL scaffolds was used because PCL scaffolds have been shown to primarily exhibit bulk diffusion related release through the amorphous regions, unlike other polymers such as PLGA that also have an erosion controlled-release component. The parameters for FITC release are defined. The other transport parameters and boundary conditions (e.g., the vascular sink) were defined as in Section 3.3.6. These parameters are for bulk diffusion-controlled release. The potential for burst release was also considered, where a percentage of the concentration in the fiber voxels (i.e., the percentage of burst release) diffused according to the diffusion rate of FITC in aqueous media.

3.3.8 Model Output

The output from the model comes in two forms. One form provides output from the summed concentration for each of the voxel types and the sinks with respect to time, as well as the number of each voxel type in the domain space. This allows
for the plotting of the total concentration per voxel type in time. Dividing the total concentration for a voxel type by the number of voxels provides an average concentration per volume in time. The benefit of using the data for this output is that it provides an easy way to see the overall trends occurring in the system. The average concentration plots can be used to compare systems that are dissimilar to one another because it normalizes the data to a per volume basis.

3.4 Results

3.4.1 CORM Activation

Activation of CORMs loaded within scaffolds is easily tracked by fluorescence and direct confirmation of CO binding can be detected by the myoglobin assay. Both, confocal light images taken of PCL/CORM–incorporated scaffolds and absorbance spectra of myoglobin assay before and after activation demonstrates the presence of CO within the electrospun fibers of our scaffolds. However, these experimental techniques do not tell us the amount of CO in real-time and what is available to the cells.

3.4.2 Experimental Validation of the Formulated Model

The representative volume (RVE) is generated from SEM images of a defined area of the electrospun scaffold, and input parameters are set within the model to generate output parameters within the three-step process model to simulate drug dose available to cells in fibrous scaffolds illustrated in Figure 3.1. The model includes a
3-step process to simulate drug doses available to cells in fibrous scaffolds. Step 1 illustrates the representative volume developed to simulate release through the entire scaffold. Step 2 shows how that a diffusion transport model is applied to the 3-D fibrous structure to predict drug release for particular voxel transitions. Step 3 provides an example of the type of output that the model provides, including total concentration for particular voxel types and a heat map depiction of drug concentration at different locations in the scaffold (the images in a row represent different z planes).

Our model results can provide insight into the effects of parameters (e.g., fiber orientation) on the concentration levels available to the cells in a simulated in vivo scenario with vasculature on one side. Multiple levels of validation were performed for the model.

3.4.2.1 Fiber Scaffold Generator

Simulated scaffolds were generated and were very similar to the experimental scaffolds used. Scaffold parameters for a 25 µm x 25 µm cross sectional area of PLGA scaffold (11% w/v with an average fiber diameter of 0.76 ± 0.36 µm) and 25 µm x 25 µm cross sectional area of PCL scaffold (22% w/v with an average 2.9 ± 0.6 µm) were created for a side-by-comparison shown in Figure 3.2A [122]. Literature values for fiber parameters were provided for these SEM images and voxel representation was accurately rendered from the original polymer fibers demonstrated in Figure 3.2B. This was shown quantitatively in Figure 3.2C as an
estimation of the volume ratio for fibers in the initial rendered scaffold in Blender to the voxel volume shows the optimal ratio of 0.14.

Figure 3.2 The model validation is shown. This included the fiber scaffold generator’s ability to accurately recreate experimental scaffolds with larger and smaller diameters as seen in this side-by-side comparison (A). Fiber parameters for these SEM images were obtained from literature values. The ability to accurately render the original polymer fibers to the voxel representation is shown visually for different angles (B) as well as quantitatively (C). An estimation of the volume ratio for fibers in the initial rendered scaffold to the voxel volume shows the optimal ratio. The fiber diameter was changed but the voxel resolution was kept constant at 0.5 μm³. Figure 2A is reprinted with permissions from [1] Bashur et al. Biomaterials, vol. 27, pp. 5681-5688, 2006 and [2] Bashur et al. Tissue Engineering, vol. 19, no. 7 and 8, 2013.
3.4.2.2 Drug Release Comparison

Experimental validation of the model was conducted on electropun PCL/FITC-incorporated scaffolds. Measurement from SEM images were used to generate the scaffold to analyze the diffusion rate out of the system for the small-molecule (FITC) (Figure 3.3A). The experimentally release profile agreed with the simulated results for pure bulk diffusion with ~0.1% burst release (Figure 3.3B). This validates the model for use with bulk diffusion-based systems such as CO.

Figure 3.3. Experimental validation of the model was performed for FITC contained within electrospun PCL scaffolds with 2% FITC loaded. An SEM image shows the fibrous structure that was recreated and used for the simulation (A). The simulated results for pure bulk diffusion and different levels of burst release are shown and compared to experimental FITC release profiles (B).
Simulated results of a sample system of CO release for a PLGA-incorporated scaffold with one cell with an RVE close to a vascular boundary is shown in Figure 3.4A. The model provided output parameters of average concentration of CO (g/cm³) as a time-dependent release profile and heat map (Figure 3.4B). Given a fiber scaffold loading 0.2% w/w CORM / PLGA, the initial concentration in the fiber would be 0.00536 g/cm³. As a result of this release value, the average concentration in the cell for this fiber scaffold at saturation levels appears to be around $1.01 \times 10^{-3}$ g/cm³.
Figure 3.4. An example CO release system for a scaffold and one cell with the RVE close to a vascular boundary (voxel size of 0.5 \(\mu\)m) (A). The output of the model includes the average concentration for interstitial fluid, fiber, and cell voxel types as a function of time (B). The insert focuses on the early time points. The concentration for individual voxels is also shown where the time is shown in each column and the z-plane is shown for each row. For each individual z-plane, the x-y area is shown.
3.4.3 Implications of the Model

Other investigations and implications of the model included the impacts of fiber orientation, fiber diameter and volume fraction on CO release for different scaffold parameters and boundary conditions. First, simulations were designed to investigate the impacts of fiber orientation. We compared simulations when fiber axes orientation was parallel to the surrounding vascular face, and when fiber axes orientation was perpendicular to it. Figure 3.5 shows that the scaffold with the fiber axes orientation parallel to the vascular face has a delayed response to vascular uptake. This means that the concentration remains in the domain longer for perpendicular when compared to parallel. Figure 3.5 shows that the fiber concentration was lower for parallel and the interstitial fluid concentration remained lower for approximately six hours. These differences result in a five percent difference in the cellular concentration levels, with parallel having a lower concentration. There is variations in CO release with changing orientation of the fibers in reference to the vascular boundary shown in Figure 3.5A, but this impact is less significant than the changes with fiber diameter demonstrated in Figure 3.5B.
Figure 3.5. Model output for CO release for different scaffold parameters and boundary conditions. The impact of the orientation of the electrospun fibers in relation to the surrounding vasculature (i.e. perpendicular and ii. parallel) on CO concentration is shown.
CO diffuses quickly and is removed from the system rapidly. Therefore, understanding the impacts of other fiber parameters such as diameter on CO diffusion is also important. Simulations of three fiber diameters were tested. The main difference in the scaffold definition between S1 and S2 is the fiber diameter. S3 has a fiber diameter that falls between the diameter used in S1 and S2, but it also has increased fiber orientation and volume fractions. One thing to note is that the increase in fiber diameter significantly reduces the number of fibers required to achieve the fiber volume fractions. This is shown in Error! Reference source not found.. The larger fiber diameter slows down the release of the CO from the fibers because it must diffuse through a longer distance in the fiber before it can enter the interstitial fluid. This is apparent by comparing the fiber concentration at \( t = 21600 \text{ s (6 hr)} \), where S2 shows that a much higher concentration is still bound up in the fibers. Larger fiber diameters result in more extended release from the fibers and more elevated concentrations in the fluid between fibers. However, the main factor that impacted the CO available to the cells was the volume fraction. Scaffolds with a larger volume fraction result in higher concentrations of CO within the cells.
Figure 3.6. Model output for CO release for different scaffold parameters and boundary conditions. The impact of fiber-fiber diameter and volume fraction variations on CO transport.
3.4.3.2 Delayed Activation Profiles

After these initial simulations, we modified the model to incorporate experimental release profiles. Experimentally, we activated DK3-incorporated PCL fibers for dry and wetted scaffolds (Figure 3.7) to determine CO release. Scaffolds were irradiated with 470nm light for 5 minutes. The CO release profile of the dry scaffold provided a higher release, similar to our observations in Chapter 2.

![DK3 Activation Curves](image)

Figure 3.7. Experimental activation release profiles for DK3 incorporated PCL fibers with 470 nm light.
We investigated the impact of a delayed activation profile on our developed model. Figure 3.8 illustrates output simulations of activation release profiles from the model after the experimental date was incorporated. Results demonstrate a reduced maximum fluid concentration of the CO, but concentrations remain higher for longer.
**Figure 3.8.** The different activation release profiles for scaffolds are shown ($t=180$ seconds). Modeling of the CO release using fitted parameters from the experimental results for dry scaffolds.
3.5 Discussion

We are confident that the delivery of CO at appropriate doses can have beneficial impacts on endothelialization and ultimately cardiovascular disease. CORMs are a promising vehicle that provides local-controlled delivery of carbon monoxide. Experimentally, we know that CO is loaded within the fibrous scaffold, released after activation, and present within the system when bound to myoglobin; however, we do not clearly understand the amount that is released to the vasculature, stays within the interstitial fluid, nor the amount that is uptaken into or leaves the cells. Studies have demonstrated that is necessary to ensure proper levels of CO because it is produced endogenously in the body, yet it can be toxic or even fatal at high levels. With this, it was imperative to better understand the transport system of CO within our tissue engineered scaffolds to avoid systemic effects. Our model provided significant information that could be used to optimize CO dose.

The biomaterial and fabrication of fibrous scaffolds can ultimately impact the drug delivery system. Fiber diameter, fiber orientation and volume fraction are all important parameters that impact cellular behavior and can be assessed by our developed model. Studies show that fiber diameter and orientation of electrospun fibers affect cell morphology, orientation, and proliferation [122]. However, it is also important to model scaffold parameters to predict the impacts on drug delivery. Our formulated model was successfully developed, validated and applied to generate output results for CO release. The output from the fiber scaffold generator produces simulated scaffolds that strongly resemble the original SEM images. The model also
provides an effective method of creating rendered objects to voxel conversions. Our model showed a loss in fidelity with large voxel sizes, as also observed in another study by Torres et al. that concluded the error was attributed to smaller voxel sizes [130]. However, there was a careful balance between creating a system with too low of a voxel resolution and too high of a voxel resolution. Therefore, we used a voxel resolution of 1:6 smaller area for all simulations, providing an acceptable rendered to voxel ratio that improved the fiber volume [124]. The simulated fiber scaffold from the fiber scaffold generator validation provided us with confidence that the output parameters were strong representation of the experimental scaffolds.

Uniquely, our model permitted the simulation of low concentrations released from microfibers. We tested the small molecule FITC within PCL as a surrogate for our expected drug CO, and we found that the release profiles are a good fit for a small percent of burst initially with the system. Most polymer systems have erosion controlled profiles for small molecules drug, but the low glass transition temperature within these PCL systems allow traditional diffusion. The experimental PCL release of FITC illustrated initial burst release and then bulk diffusion, which is the expected release profile for CO. After validation, our model output for CO demonstrated that the initial changes in the system happen rapidly as expected due to the fast nature of CO diffusion in the scaffold. The initial concentration is bound up in the surrogate FITC fibers and released quickly into the interstitial fluid, creating a spike in the interstitial concentration within the first five seconds of starting the simulation, after which the system begins to level off. The effects of the surrounding vasculature
become apparent, and by about 10 minutes, the system is mostly driven by the loss through cellular consumption and to the surrounding vasculature. Our model was able to demonstrate bi-phasic levels of release, and we expect this to be relevant for gasotransmitters within our delivery system and modeling native blood vessels.

Our model allows for the prediction of our drug release from a conduit grafted into the blood vessels. It is ideal to understand the function and performance of the biomaterial prior to implantation or use within the native environment, and the \textit{in vitro} studies can give some enlightenment, but it is not a true direct comparison of what takes place within the \textit{in vivo} condition. With our model, we are permitted to readily modify boundary conditions to simulate real world applications such as flow within blood vessels.

The featured biomaterial scaffold can achieve cell orientation through contact guidance and can regulate cell attachment and cell spreading \cite{131,132}. The designed scaffold will impact the cellular behavior (e.g. spreading). We hypothesized the model will demonstrate how much CO gets into the cell. Determining the effects of this concentration at the cellular level is difficult. Studies show that the borderline safe level of CO inhalation is approximately 500 ppm \cite{35}; however, translating this value to a dosage at the cellular level is complicated. In fact, a search of the literature returned no methods to derive cellular concentration from an inhalation quantity (for CO or any suitable surrogates). Our model implication results for fiber diameters gives insight that release from larger diameter fibers have decreased release rate, slower release into the fluid and sustained increase of CO concentration. More
importantly, other implications suggested that scaffolds with a higher volume fraction result in higher release rate from fibers and an increase in cellular concentration (60% higher). This is the most important variable to understand when predicting the dose of a gasotransmitter delivered to cells because it implies that cells that are more spread have more cell-material interactions, and more drug gets into the cells.

3.6 Conclusions

We demonstrate that the validated model can be used to predict drug availability to cells for a variety of scaffolds and drug molecules. For CO release, this model is necessary because of the limitations with real-time experimental analysis. Our results suggest that only a fraction of the initial concentration of gasotransmitters released from fibers that enters the interstitial fluid in vivo, or culture media in vitro, will be available to cells. We also demonstrated that fiber diameter and fiber density are important parameters not just for traditional tissue engineering, but also for drug delivery. In our future work, we hope that further development of this model will provide insight into understanding of hindered diffusion and the correlation between inhalation levels and cellular concentrations of CO.
3.7 Acknowledgments

This study was supported by a National Science Foundation Grant (No. CBET 1510003) awarded to Chris A. Bashur. Kai Clarke and Kellen Maurus provided valuable assistance with the experimental release profiles.
Chapter 4: Conclusion and Future Work

4.1 Conclusion

The overall goal of this dissertation was to generate a CORM-loaded electrospun scaffold that can be used to develop a small diameter vascular graft, and to determine the impacts of CO on ECs which are part of a functional endothelium. Towards this goal, two complimentary steps were performed: (1) the investigation of CO-loaded electrospun scaffolds impacts on ECs through in vitro studies for cardiovascular tissue engineering, and (2) the determination of mass transport of CO through diffusion-based modeling and drug delivery of gasotransmitters from tissue engineered scaffolds.

The first study was to determine toxicity, biocompatibility and effect of CO on endothelial cells to develop a functional endothelium. We accomplished this by seeding spin-coated thin films and electrospun PCL scaffolds at varying concentrations of CORMs. They were fabricated, characterized, seeded with endothelial cells, cultured up to 14 days, and analyzed with biochemical assays and immunofluorescence stains. The results demonstrated that DK3 increases CO release from scaffolds compared to other DK compounds. This included an extended maximum incubation time of at least 24 hours. We have shown that both the CORM (DK3) material itself and CORM activation and CO release is non-toxic and allows EC proliferation, spreading, and functional marker expression (e.g., von Willebrand Factor (vWF)) from biocompatibility results. In addition, we found that endothelial cell proliferation is in a direct relationship with seeding density on standard TCPS,
but fewer cells attached on the PCL surfaces. This lets us know that there should be optimization of our scaffold material similar to the treated TCPS surface to achieve ideal attachment and proliferation. However, we can still conclude that there is proliferation of endothelial cells when seeded on DK3 scaffolds at both day 3 and day 14. We also conclude that DK4 is not toxic at the levels tested. Lastly, we also demonstrate that the CORM-loaded scaffold can be grafted and remain patent in small diameter vessels.

The second task was to simulate the mass transport of CO within our drug delivery system and to validate our computational model. This was completed through the design and development of a diffusion-based computational model. Electrospun scaffolds and experimental release profiles were very similar to the model output of simulated scaffolds and drug release profiles, demonstrating successful validation. Results demonstrate that the validated model can be used to predict drug availability to cells for a variety of scaffolds and drug molecules. Our results suggest that only a fraction of the initial concentration of gasotransmitters released from fibers that enters the interstitial fluid in vivo, or culture media in vitro, will be available to cells. We also demonstrated that fiber diameter and fiber density are important parameters not just for traditional tissue engineering, but also for drug delivery.

Through the tasks performed in this research project several important results were achieved: (1) development of a more hydrophobic CORM-loaded scaffold, (2) showing that CORMs (DK3 and DK4) do not exhibit toxicity to ECs at all
concentrations, and (3) computational modeling simulations can predict CO dose available to cells on the microfiber level.

4.2 Future work

While the results of this research projects are promising, there are some important areas where further research is required. Three of these areas include: (1) performing more cellular studies with varying coatings such as fibronectin, laminin, collagen to promote better cell attachment and function on polymer scaffolds, and (2) using and applying the model to predict appropriate dose of CO of our tissue engineered strategy.

4.2.1 Surface modifications for improvement of EC function

The first area of future work is to perform surface modifications to our scaffold material to promote cell attachment, migration, and proliferation. In addition, cellular studies will be completed to determine specific dose impacts of CO on endothelial cells after the surface coating is optimized. Studies have shown the need to modify the surface of electrospun PCL nanofibers to improve their compatibility with endothelial cells, and to show the potential application of PCL nanofibers as TEVGs [133]. The results presented in Chapter 2 of this dissertation further support this by demonstrating the limitations of EC attachment, migration, and proliferation on non-treated surfaces. These results are due to our polymer
materials being hydrophobic and not easily allowing protein absorption which is required for cell adhesion via integrins. It is known that the cellular behavior such as migration, proliferation and differentiation could be regulated by the extent of initial cell attachment. The size and shape of cell spreading area, as well as the number, size, shape and distribution of focal adhesion plaques are decisive for further migratory, proliferative and differentiation behavior of anchorage dependent cells. If this extent is very small (i.e. attachment of round cells without formation of focal contacts and spreading), the cells usually do not survive [134]. Therefore, conducting surface modifications to the electrospun scaffold is an important parameter to improve cell-material interactions.

Future work can be performed to improve cellular behavior function including attachment, migration, and proliferation to provide a better understanding of CO-dose impacts on ECs. One strategy to accomplish this involves coating the electrospun scaffold with ECM proteins such as fibronectin or laminin. The experiments can be performed to test EC attachment to varying doses of CO-loaded thin films. We will spin coat thin films of PCL/DK4 and control PCL on separate glass coverslips to demonstrate cell attachment on 2-D surface. We will also coat the same mentioned conditions in fibronectin and laminin to determine the best coating to optimize cell behavior. We will seed ECs \((n=3)\) on coated surfaces, irradiate (5 minutes of 470nm light) the activated conditions 24 hours after seeding, and verify cell number with the AlamarBlue assay, DNA assay and PCR at Day 1, 3, 7, and 14. This experimental plan reflects the toxicity and biocompatibility studies in Chapter
2, but the surfaces would be coated and the PCR assay will also provide results on CO-dose impacts on ECs.

This future work is expected to show that deposition of ECM proteins (fibronectin or laminin coating) are necessary for EC cell behavior resulting in an increase of cell attachment number, migration, and proliferation. After analysis of all conditions and assays, we expect to determine the best surface modifications and understand impacts of CO on endothelial cells. These findings will offer important information needed to further test DK4/PCL electrospun scaffolds that will be used in the next in vivo studies. Overall, this future plan is expected to also provide the appropriate CO dose levels for our TEVGs.

4.2.2 Applying the model to predict appropriate dose of CO drug delivery systems

The second area of future work is to implement the computational model developed in Chapter 3 to predict appropriate dose of CO from our drug delivery systems. We’ve discussed CO delivery when released from CORMs after surface modifications and CO delivery released from DK incorporated nanoparticles. These are separate drug delivery systems, but it very important to understand drug release from both strategies for treatment of CO. Computational modeling of surface modified DK4 thin films will be set up as a simple diffusion system as discussed in Chapter 3. The extra layer of absorbed ECM protein of fibronectin or laminin will not add complexity to the model input. For example, we know from our results in Chapter 3 the more interaction between cells and fibers, (e.g. with cell spreading)
will allow more drug into the cells. Therefore, with the surface modifications, we expect to see an increase in cell attachment, and overall endothelial cell function. This should correlate to more ECs well spread on our scaffold material and it is important to determine CO dose delivery into these cells with the increase in cell spreading and cell density.

Another strategy discussed in Chapter 2 was using DK4 incorporated nanoparticles for CO delivery as a therapeutic molecule. Nanoparticles have shown promise as both drug delivery vehicles and direct anti-tumor systems, but they must be properly designed in order to maximize efficacy [135]. Therefore, computational modeling should be used to validate the release of drugs from nanoparticles. Modeled processes will include the release of drugs at the diseased site and the physical interaction between the nanoparticle and cells. Most CORM compounds are internalized within cells and the effect found can be more than the attribution of CO alone. It is important to note that drugs can either be encapsulated or attached to nanoparticles; therefore, we will investigate the release of CO incorporated within nanoparticles.

This future work is expected to show that computational modeling can be used to better predict drug release from various delivery systems. We expect to see the output of our model illustrate an increase in cellular uptake because more cells are assumed to be more spread after surface modifications on thin-films and electrospun scaffolds. With this, we further expect there will be more significance in dose impacts of CO on ECs. Modeling DK3 incorporated NPs will predict release as
we progress towards testing in vivo because environmental differences also impact
drug delivery. Currently, NPs are not used widely in the pharmaceutical industry due
to their poor predictability, but this future work may offer better predictability for
therapeutic uses in the field of biomedical engineering.

7.2.3 Final Conclusions

The focus of this research project was to generate a CORM-loaded
electrospun scaffold to develop a small diameter vascular graft and to determine the
impacts of CO on ECs for a functional endothelium. Through this project, we
investigated the impacts of CORM-loaded thin films on ECs through in vitro studies,
and determined the mass transport of CO through diffusion-based modeling and drug
delivery of gasotransmitters from tissue engineered scaffolds. Prior results suggested
limited cell proliferation due to low initial cell attachment on PCL surfaces.
Important areas of future work include performing surface modifications on the
polymer scaffold to improve cell attachment, determining appropriate dose after
material has been modified, and modeling the drug delivery systems discussed in this
Chapter 2. Successful completion of these next steps is envisioned to result in a
CORM-loaded electrospun scaffold that will be suitable for in vivo testing as
TEVGs.
References


Appendix

A.1: List of Articles with Collaborative Efforts


Carbon monoxide (CO) is a gasotransmitter that plays important roles in regulating cell functions and has shown therapeutic effects in clinic studies. CO releasing molecules (CORMs), which allow controlled release of CO in physiological conditions, have been intensively studied in the past decade. While most CORMs are metal complexes, several nonmetallic CORMs have also been developed, and most of them were reported in recent years. The major advantages of nonmetallic CORMs are potentially low toxicity and easy modification for property tuning. Syntheses, CO-release mechanisms, biological behaviors, and physicochemical properties of these nonmetallic CORMs are reviewed here. The first part of this short review covers the nonmetallic CORMs that do not require irradiation to release CO, which includes methylene chloride, CORM-A1 and its derivatives, amine carboxyboranes, and bimolecular CORMs. The second part focuses on the CORMs that release CO under irradiation (PhotoCORMs) including unsaturated cyclic diketones, xanthene carboxylic acids, meso-carboxy BODIPYs, and hydroxyflavones. Future prospects are discussed at the end of this review.

My role included all experimental assays in toxicity and viability tests.

Carbon monoxide (CO) is a gasotransmitter that has shown therapeutic effects in recent studies. Photo carbon monoxide releasing molecules (PhotoCORMs) allow the delivery of CO to be controlled by light. In this work, a new organic photoCORM DK4 is studied. DK4 is a diketone type photoCORM, which releases two CO molecules under visible light and simultaneously generates a fluorescent anthracene derivative. However, this type of CORM suffers from a deactivating hydration reaction and often needs to be incorporated in polymers or micelles. The two highly hydrophobic tert-butyl groups of DK4 protect it from the hydration reaction. DK4 functions in 1% DMSO aqueous solution, in which other DKs are deactivated. DK4 was incorporated in a poly(butyl cyanoacrylate) (PBCA) nanoparticle. PBCA has been used as tissue adhesive and has been extensive studied for brain delivery of drugs. The PBCA/DK4 nanoparticle showed good photoactivity and low cytotoxicity, and thus is a promising material for studying the biomedical effects of CO.

My role in this study included all biological experiments including cell culturing, seeding, Alamar Blue and DNA assay, data analysis, and writing of the experimental results.
Several clinical situations including birth defects, trauma, and non-union fractures often result in critically sized defects that require a graft that can remodel and integrate with the existing bone as well as mitigate the risk of infectious complications. Delivery of gasotransmitters from tissue engineering scaffolds is a potential option to provide antibacterial properties while simultaneously promoting osteogenesis and tissue vascularization. Gasotransmitters, such as nitric oxide, carbon monoxide, and hydrogen sulfide, are inorganic gases that have an important role in cell signaling and supplemental doses have also been shown to provide bactericidal properties. This chapter will review the importance of understanding the complex, and dose-dependent, impacts of different gasotransmitters on both bacterial and mammalian cells. The current research into the selectivity of a gasotransmitter dose for killing bacteria compared to mammalian cells is a particular focus. The chapter will also discuss the applications of gasotransmitters to engineered tissues, with a focus on bone and vascular, as well as the current limitations for incorporating gasotransmitters within scaffolds that need to be addressed.
Appendix B: Short Description of Collaborative Ongoing Projects

**B.1.1 Blended Electrospun Meshes with Low Amounts of Collagen Type 1 are Hemocompatible**

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I. Introduction

Blended scaffolds made with natural and synthetic biomaterials are often used in vascular applications. We saw this with our previous studies where 10% collagen within tissue engineered vascular grafts (TEVGs) reduced gene expression of the macrophage marker *Cd68* and appeared to reduce CD80 protein expression in images after 6 weeks of grafting, yet still exhibited mechanical integrity [1]. In addition, it was noteworthy that there were no concerns with thrombosis since collagen type 1 is pro-thrombogenic. Thus, the goals of this study were to systematically investigate the impact of collagen ratio on *in vitro* hemocompatibility and characterize the material to investigate the biomaterial mechanism for the observed results. Hemocompatibility comparable to ePTFE surfaces was demonstrated for collagen type 1 / PCL meshes with ≤ 25% collagen [2].
II. Materials and methods

For hemocompatibility, meshes were prepared by electrospinning 0, 10, 25, and 50% (w/w) blends of collagen/PCL dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol. Thick sheets were incubated in rat platelet-rich plasma, dried, and analyzed with SEM to semi-quantitatively characterize platelet adhesion and activation. Testing of the meshes and controls also included hemolysis. For characterization, blends of collagen type 1 and PCL were electrospun on glass coverslips to clearly observe individual fibers. Sample were analyzed with polarized light both with and without a lambda plate to semi-quantitatively characterize the material crystallinity, including the degree of crystallinity and crystal orientation. Samples were also stained with picrosirius red to assess the presence of collagen.

III. Results and Discussion

Interestingly, our hemocompatibility testing revealed that 10% and 25% collagen electrospun meshes had significantly lower platelet adherence than 50% collagen and comparable or lower levels than pure PCL ($n = 3$; one-way ANOVA with Tukey). Additionally, 10% and 25% collagen samples had more activated platelets (e.g., dendritic) than 50% collagen or pure PCL. Only 50% collagen exhibited large platelet aggregates, indicating a difference on these surfaces. These results are shown in Figure 1 and Figure 2.
Figure 1. SEM images at 2,000X showing platelet adhesion on electrospun meshes of (A) pure PCL, (B) 10% collagen/PCL, (C) 25% collagen/PCL, and (D) 50% collagen/PCL. White arrows show some of the adhered platelets.
The levels of activation for platelets on the different materials can be visualized in (Fig. 3 and 4). These results agree with a previous study that found significant thrombosis in vivo with 50\% collagen/PCL blends [2]. Since collagen I is pro-thrombotic, it is important to investigate why lower levels of collagen exhibited good hemocompatibility.

**Figure 2.** Average number of platelets/ mm$^2$ in electrospun meshes of pure PCL, 10\% collagen, 25\% collagen, and 50\% collagen. Significance was calculated with one-way ANOVA and Tukey multiple comparisons ($p<0.05$) for $n = 3$. (*) Significance from 50\% collagen/PCL.
Figure 3. SEM images at 5,000X highlighting levels of platelet activation and extensions on electrospun meshes for (A – B) 50% collagen/PCL, and (C) Platelet adhesion on glass positive control. Colors showing increasing levels of activation: Non-Dendritic, Dendritic, Spread Dendritic, Fully Spread, Aggregate.
Our picrosirius red staining demonstrated increased birefringence in fibers with low collagen percentages indicating that collagen was present on the fiber surface for platelet interaction shown in Figure 5. However, the results suggest that percent collagen within the fibers impacts PCL crystallinity as a systematic decrease in birefringence and an increase in changes in crystal direction was observed when collagen concentration was increased from 0% to 50%. This indicated a more random orientation of individual PCL crystals.

Figure 4. Characterization of platelet activation in electrospun meshes. Only 50% collagen had large aggregates. There were more dendritic than non-dendritic platelets for pure PCL and 50% collagen, indicating more activation than low collagen percentages.
We are also investigating different macroscale fiber orientations and fiber diameters to determine their impact on electrospun mesh crystallinity. To further investigate the biomaterials mechanism, we are currently investigating the distribution of collagen with TEM.

Figure 5. Polarized light images at 40X magnification of 10% collagen/PCL (A) without and (B) with picrosirius staining. 25% collagen/PCL (C) without and (D) with picrosirius staining. (E) Polarized light image at 100X oil immersion magnification showing dotted fiber in 25% collagen/PCL mesh. White arrows show fibers with areas where crystallinity direction changes.
IV. Conclusion

The aforementioned hemocompatibility results combined with our previous results [1], suggest that the balance between collagen and synthetic polymers can have an important impact on TEVG viability. This impact cannot be explained by the lack of collagen on the surface since it is still present with lower collagen ratios. It is clear that different collagen ratios impacted the PCL crystallinity, but also potentially the collagen structure and its distribution. These results are also likely relevant for other natural / synthetic blend meshes used as biomaterials.

This finding, combined with results from our previous study [1], suggest that the balance between collagen and synthetic polymers can have an important impact on TEVG viability. This impact cannot be explained by the lack of collagen on the surface with lower collagen ratios, since it is present. It is clear that the different collagen ratios impacted the PCL crystallinity, but also potentially the collagen structure and its distribution. These results are also likely relevant for other natural / synthetic blend meshes used as biomaterials.
References:
