Summer 8-15-2015

Synthesis of Clickable Poly(ethylene glycol) Derivatives for Fabrication of Modular Microsphere-Based Scaffolds to Promote Vascularization

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Synthesis of Clickable Poly(ethylene glycol) Derivatives for Fabrication of Modular Microsphere-Based Scaffolds to Promote Vascularization

by

Peter Khoatan Nguyen

A dissertation presented to the Graduate School of Arts & Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

August 2015
St. Louis, Missouri
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Acknowledgments

I would like to foremost sincerely thank my advisor, Donald Elbert, for giving me the opportunity to pursue my PhD in his lab. I would also like to thank him for all his guidance and support throughout my long PhD career. His intellectual knowledge and creativity and his patience with me helped me through the challenges in my graduate training. His mentorship has been essential in my development as a scientist and critical thinker.

I would like to thank my thesis committee (Shelly Sakiyama-Elbert, Garland Marshall, Robert Mecham, and Steven George) for taking the time to evaluate my work and offering useful advice to help me achieve my goals. Their support helped me overcome the challenges that I faced along the way.

I would like to thank all the members who have come and gone from the Elbert Lab for creating a wonderful working environment throughout the years. Their collaboration and friendship was much appreciated during my graduate school journey. I would like to specifically thank Casey Donahoe, Megan Flake, Jacob Roam, Amanda Smith, Evan Scott, Michael Nichols, Lily He, and Kalyani Kulkarni. They provided help when it was needed and engaged in great discussions that were not always related to science. I would like to especially acknowledge Casey Donahoe for being highly entertaining and keeping me going in the right direction, and Jacob Roam for helping me run the lab these last couple of years. I would also like to thank all the undergraduates I mentored and worked with throughout the years, especially Jason Shields, Chris Snyder, Debra Yen, and Ian Kinstlinger.

I would like to thank the members of the Sakiyama-Elbert Lab for contributing to the wonderful work environment as a second lab family and for the help and friendship they offered.
over the years. I would also like to thank the faculty and staff of the Biomedical Engineering
department including Frank Yin, Glen Reitz, Amanda Carr, and Karen Teasdale for their
friendship, support, and help with logistical items.

I would like to thank all my past collaborators who helped me with my work specifically
Matthew MacEwan for helping me with scaffold implants in mice, Li Li for helping me image
vascularization in scaffolds, and Venktesh Shirure for helping me with scaffold vascularization.
I would also like to thank my funding sources: The National Institutes of Health and Washington
University in St. Louis.

Finally, I would like to thank God, my family, and my friends for all of their love and
support throughout my life. To my parents, Yen Nguyen and Nhun Tran, thank you for always
supporting me with your love and encouraging me to work hard in all my endeavors. To all my
friends, especially the ones I made in St. Louis, thanks for bringing great friendship, fun, and
excitement into my life. To my fiancée, Maria Praggastis, your constant love and support
through thick and thin has made this journey towards my PhD much more enjoyable. I dedicate
this work to all of them as a token of my appreciation.

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August 2015
Vascularization plays an important role in supporting transplanted tissues and cells in tissue engineering applications. Most tissues require access to blood vessels for the delivery of oxygen and nutrients, as well as the removal of carbon dioxide and cellular waste products. Without an adequate blood supply, cells within tissue-engineered constructs and scaffolds lose viability and fail to perform their intended functions. The goal of this dissertation was to design scaffolds that can promote vascularization of biomaterial implants for biomedical applications. In order to accomplish this goal, clickable poly(ethylene glycol) (PEG) derivatives were synthesized in order to fabricate modular microsphere-based scaffolds that could deliver angiogenic growth factors and molecules. Microspheres were formed by lowering the lower critical solution temperature (LCST) of PEG, inducing thermal phase separation, and forming spherical PEG-rich domains that increased in size until they reached the gel point. Using a PEG/dextran aqueous two-phase system, microspheres were crosslinked via click reactions to form scaffolds. Conjugation of these scaffolds with cell adhesion peptides and proteins allowed for cell attachment and migration. Subsequently, scaffolds were formed in the presence of cells to confirm cell attachment and demonstrate that the functionalized PEG and the scaffold
fabrication process did not affect cell viability. To show that the scaffolds did not adversely affect the activity of loaded growth factor, biologically active vascular endothelial growth factor (VEGF) was successfully loaded into and delivered from the scaffolds in an endothelial cell migration assay. Finally, fibroblasts and endothelial cells were seeded into the scaffolds to determine if the scaffolds were able to support vascular formation. After 21 days, the scaffolds demonstrated small regions of vascularization. The vascularization in these scaffolds was comparable to what we had observed with similar scaffold implants in mice. To improve scaffold vascularization, future work will be done to adjust their porosity, growth factor delivery, and introduce scaffold degradability. Overall, this dissertation demonstrated that clickable PEG microsphere-based scaffolds have excellent potential for inducing vascularization in tissue engineering applications.
Chapter 1

Introduction

1.1 Overview and Objectives of Dissertation

Providing a robust vascular network is required for the survival of transplanted and engineered tissues, as the diffusion of oxygen and nutrients alone is not adequate for cell survival in thicker tissues or tissue-engineered constructs (Rouwkema, Rivron et al. 2008; Lovett, Lee et al. 2009; Phelps and García 2010; Novosel, Kleinhans et al. 2011; Chu and Wang 2012; Auger, Gibot et al. 2013; Birbrair, Zhang et al. 2014). Most tissues require a highly branched system of larger blood vessels and smaller capillaries to supply oxygen and nutrients (Rouwkema, Rivron et al. 2008; Lovett, Lee et al. 2009; Phelps and García 2010; Novosel, Kleinhans et al. 2011; Chu and Wang 2012; Auger, Gibot et al. 2013; Birbrair, Zhang et al. 2014). The tissues that do not require a dedicated vascular system, namely skin, cartilage, and cornea, are supplied with oxygen and nutrients from diffusion through a nearby system of blood vessels and capillaries (Rouwkema, Rivron et al. 2008; Lovett, Lee et al. 2009; Phelps and García 2010; Novosel, Kleinhans et al. 2011; Chu and Wang 2012; Auger, Gibot et al. 2013; Birbrair, Zhang et al. 2014). The diffusion limit of oxygen limits the maximum distance of blood vessels and capillaries from tissues to 200 µm. However, implanted tissues are usually located at an insufficient distance from those blood vessels and capillaries (Rouwkema, Rivron et al. 2008; Lovett, Lee et al. 2009; Phelps and García 2010; Novosel, Kleinhans et al. 2011; Chu and Wang 2012; Auger, Gibot et al. 2013; Birbrair, Zhang et al. 2014). To overcome the diffusion limit,
several strategies can be employed to provide blood vessels and capillaries to the tissues, including cell-based angiogenesis and scaffold based strategies (Rouwkema, Rivron et al. 2008; Lovett, Lee et al. 2009; Phelps and García 2010; Novosel, Kleinhans et al. 2011; Chu and Wang 2012; Auger, Gibot et al. 2013; Birbrair, Zhang et al. 2014). The objective of this dissertation is to create scaffolds with poly(ethylene glycol) (PEG) microspheres that can support a vascular network to provide a blood supply to transplanted tissues and tissue-engineered constructs.

We approached this problem with a scaffold based strategy that also incorporates concepts from cell-based angiogenesis. Scaffolds are formed in a modular manner, and they require the synthesis of several different PEG derivatives (Nichols, Scott et al. 2009; Scott, Nichols et al. 2010; Smith, Segar et al. 2012; Nguyen, Snyder et al. 2013). Each PEG derivative has a different functional end group, which allows for complementing PEG derivatives to crosslink together to form PEG microspheres (Nichols, Scott et al. 2009; Scott, Nichols et al. 2010; Elbert 2011; Elbert 2011; Smith, Segar et al. 2012; Nguyen, Snyder et al. 2013). We developed a method to synthesize PEG derivatives with two functional end groups per PEG molecule. These multifunctional PEG derivatives served the same purpose for creating PEG microspheres, but had greater versatility with two functional groups. PEG microspheres can then be crosslinked together to form a scaffold upon centrifugation (Scott, Nichols et al. 2010; Elbert 2011; Elbert 2011; Smith, Segar et al. 2012; Nguyen, Snyder et al. 2013). Different types of microspheres can be incorporated during centrifugation to create modular scaffolds with different properties (Scott, Nichols et al. 2010; Smith, Segar et al. 2012; Nguyen, Snyder et al. 2013). Incorporating degradable microspheres (porogenic microspheres) increases the porosity of the scaffolds while introducing other microspheres with special functional groups allows for the incorporation of cell adhesion molecules and proteins to promote cell attachment (Scott,
Nichols et al. 2010; Smith, Segar et al. 2012; Nguyen, Snyder et al. 2013; Roam, Nguyen et al. 2014). The versatile nature of the scaffolds also provides the option for modification with proteins and molecules for controlled drug delivery through affinity interactions (Wacker, Scott et al. 2006; Scott, Nichols et al. 2010; Roam, Nguyen et al. 2014). Modifications that can be introduced include the addition of RGD peptide, laminin, heparin, and bovine serum albumin (BSA). In addition to their versatility, these scaffolds are designed to prevent unwanted cell and protein interactions under physiological conditions through the use of functional groups that undergo specific reactions, such as bioorthogonal click reactions (Iha, Wooley et al. 2009; Nwe and Brechbiel 2009; Nguyen, Snyder et al. 2013). By combining versatility of the PEG derivatives with the modular scaffold fabrication process, porous PEG scaffolds were formed that could provide controlled release of growth factors for vascularization. To assess the effectiveness of these scaffolds for vascularization, they were either implanted subcutaneously into mice or co-cultured with fibroblasts and endothelial cells.

1.2 Vascularization

Without blood vessels, tissues cannot grow beyond the diffusion limit of oxygen, which is 100-200 µm (Carmeliet and Jain 2000; Rouwkema, Rivron et al. 2008; Novosel, Kleinhans et al. 2011; Auger, Gibot et al. 2013). Blood vessels not only provide cells and tissues access to oxygen, but they also provide nutrients and remove carbon dioxide and cellular waste products (Carmeliet and Jain 2000; Rouwkema, Rivron et al. 2008; Novosel, Kleinhans et al. 2011; Auger, Gibot et al. 2013). While larger tissue-engineered constructs can be supplied with
nutrients in vitro with the aid of perfusion bioreactors, new blood vessel formation is still required for tissue-engineered constructs and scaffolds to be successful post implantation (Portner, Nagel-Heyer et al. 2005; Janssen, Oostra et al. 2006; Rouwkema, Rivron et al. 2008). Since implants tend to be greater than 200 µm, capillary networks need to be formed within the implants. Following the implantation of scaffolds and tissue-engineered constructs, blood vessels from the host invade to form a vascular network as a response to the implant (Rouwkema, Rivron et al. 2008). This type of vascularization, in which new blood vessels form from preexisting vascularization, is referred to as angiogenesis (Auger, Gibot et al. 2013; Birbrair, Zhang et al. 2014). Angiogenesis is initiated by growth factor activation of endothelial cells, causing basement membrane degradation by proteases and endothelial cell migration and proliferation toward the angiogenic stimuli (Carmeliet and Jain 2011). This is followed by sprout formation and extension, lumen formation, and vessel maturation with pericytes (Carmeliet and Jain 2011). New formation of blood vessels without any preexisting vasculature is referred to as vasculogenesis, and it normally occurs during embryologic development (Auger, Gibot et al. 2013). Vasculogenesis is similar to angiogenesis but begins with growth factor activation of angioblasts and haemangioblasts, followed by growth factor mediated vessel assembly, and finally activation of receptors to transition into angiogenesis (Demir, Seval et al. 2007). Despite the spontaneous blood vessel ingrowth that occurs in conventional implants, the complete vascularization takes several weeks because vessel growth is limited to tenths of micrometers a day (Clark and Clark 1939). Moreover, slow vascularization may lead to non-uniform cell integration, with higher cell densities within the outer regions of the scaffold (Malda, Rouwkema et al. 2004). Successfully promoting quick and complete vascularization in
biomaterial scaffolds and tissue-engineered constructs will expand scaffold applications in tissue engineering.

Many growth factors and bioactive molecules come into play for promoting different stages of angiogenesis. Some of the growth factors and bioactive molecules involved in promoting vascularization include angiopoietin-1, angiopoietin-2, fibroblast growth factor, platelet-derived growth factor, vascular endothelial-cadherin, sphingosine-1-phosphate (S1P), and vascular endothelial growth factor (VEGF) (Conway, Collen et al. 2001; Wacker, Scott et al. 2006). The two that we will focus on for drug delivery in this work are S1P and VEGF.

1.2.1 Sphingosine-1-Phosphate

The bioactive lipid S1P is an appealing biological factor because it has been shown to be a powerful and complete angiogenic regulator of endothelial cell function, promoting cell survival, proliferation, migration, barrier integrity, and angiogenic differentiation (Liu, Wada et al. 2000; Allende, Yamashita et al. 2003; McVerry and Garcia 2005; Wacker, Scott et al. 2006; Lucke and Levkau 2010). By interacting with the family of G protein-coupled receptors called S1P receptors (formally known as endothelial differentiation gene (Edg) receptors), S1P can trigger key endothelial cell behaviors that improve vascularization (Lee, Van Brocklyn et al. 1998; Liu, Wada et al. 2000; Allende, Yamashita et al. 2003; Kono, Mi et al. 2004; Lucke and Levkau 2010). S1P has been shown to promote endothelial cell migration during platelet activation (English, Welch et al. 2000), and cause a dose-dependent enhancement of endothelial cell migration (Liu, Verin et al. 2001). At physiological concentrations, S1P has been shown to
inhibit smooth muscle cell migration (Boguslawski, Grogg et al. 2002; Tamama, Tomura et al. 2005). S1P may also inhibit recruitment of endothelial cells by decreasing their permeability, stabilizing intracellular junctions in a concentration-dependent manner (McVerry and Garcia 2005; Gavrilovskaya, Gorbunova et al. 2008; Tengood, Kovach et al. 2010). Research has shown that S1P1 receptor knockout mice exhibit a lethal phenotype with severe hemorrhaging, indicating that S1P is required at later stages of angiogenesis for vessel maturation (Liu, Wada et al. 2000; Allende, Yamashita et al. 2003; Kono, Mi et al. 2004). Controlling the release of S1P is therefore crucial to enhancing vascularization.

### 1.2.2 Vascular Endothelial Growth Factor

Another desirable factor for vascularization is VEGF because of its association with promoting angiogenesis and endothelial cell migration. Mammals have five members in the VEGF family: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PLGF) (Holmes, Roberts et al. 2007). In angiogenesis, VEGF normally refers to VEGF-A due to its importance in vascular development (Holmes, Roberts et al. 2007). More specifically, VEGF has been shown to play an important role as an endothelial cell specific growth factor that stimulates endothelial cell migration, proliferation, and nitric oxide release, allowing angiogenesis to take place (Conway, Collen et al. 2001; Kliche and Waltenberger 2001; Barrientos, Stojadinovic et al. 2008). The effects of VEGF are mediated mainly by two tyrosine kinase cell receptors, VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2), that trigger signaling pathways upon binding VEGF (Kliche and Waltenberger 2001). The role of VEGFR-1 in angiogenesis is not completely known. However, VEGFR-1 may moderate the amount of free
VEGF available to bind to VEGFR-2 and prevent vessel overgrowth by competing with VEGFR-2 binding (Carmeliet and Jain 2011). VEGFR-2 is the principal mediator of endothelial cell proliferation, migration, survival, and permeability (Holmes, Roberts et al. 2007). In contrast to S1P, VEGF has been shown to be important at early stage angiogenesis by upregulating matrix metalloproteinase (MMP)-2 and MMP-9, which results in the destruction of the extracellular matrix and destabilizes the blood vessels, promoting vessel infiltration (Sang and Douglas 1996; Sang 1998; Bouis, Kusumanto et al. 2006; Gavrilovskaya, Gorbunova et al. 2008; Tengood, Kovach et al. 2010). Managing the temporal release of VEGF will play an important part in improving vascularization in scaffolds.

1.3 Drug Delivery

Drug delivery refers to the systems and technologies engineered to transport therapeutic agents to specific targets. It is often used to deliver growth factors and bioactive molecules to elicit biological responses, such as angiogenesis, in cells and tissues. Choosing the appropriate growth factors and bioactive molecules and devising a suitable delivery strategy are the main aspects of successful drug delivery. With respect to promoting and enhancing angiogenesis of tissue-engineered constructs and scaffolds, drug delivery systems must deliver growth factors and bioactive molecules to destabilize existing blood vessel walls and induce endothelial cell proliferation and migration to form sprouts and tube structures (Wacker, Scott et al. 2006). To optimally enhance vascularization, the complete biological response of vascular cells to biological factors over time must be addressed. Therefore, not only are the types of factors that
are delivered relevant, but how they are delivered temporally are important considerations that need to be made.

1.3.1 Importance of Controlled Release of S1P and VEGF

The uncontrolled release of S1P and VEGF can potentially inhibit vascularization in scaffolds instead of promoting it. Determining when to deliver each factor is critical, since the process of angiogenesis is organized into a series of events that begin with vessel destabilization, followed by endothelial cell proliferation and migration, sprout and tube structure formation, and finally vessel maturation (Bouis, Kusumanto et al. 2006; Wacker, Scott et al. 2006). As mentioned previously, VEGF is involved in the early stage of angiogenesis while S1P is required for the later stage of angiogenesis (Sang and Douglas 1996; Sang 1998; Liu, Wada et al. 2000; Allende, Yamashita et al. 2003; Kono, Mi et al. 2004; Bouis, Kusumanto et al. 2006; Gavrilovskaya, Gorbunova et al. 2008; Weiss, Zimmermann et al. 2009; Tengood, Kovach et al. 2010). Delivering too much VEGF during the latter stages of angiogenesis results in the destruction of the extracellular matrix and destabilization of blood vessels (Sang and Douglas 1996; Sang 1998; Bouis, Kusumanto et al. 2006; Gavrilovskaya, Gorbunova et al. 2008; Tengood, Kovach et al. 2010), and elevated levels of S1P during early and middle stages of angiogenesis reduces endothelial cell recruitment and migration (McVerry and Garcia 2005; Gavrilovskaya, Gorbunova et al. 2008; Tengood, Kovach et al. 2010). More importantly, these factors play multiple roles in the body (Arsic, Zacchigna et al. 2004; Sattler and Levkau 2009), and the unchecked release of factors from scaffolds could lead to other side effects.
1.3.2 Strategies for Controlled Release

A delivery system that releases VEGF and S1P sequentially may provide optimal conditions for vascularization in PEG scaffolds. The main categories of delivery systems include diffusion-based, affinity-based, swelling-based, erosion-based, and stimuli-based (Alarcon, Pennadam et al. 2005; Oupicky, Bisht et al. 2005; Arifin, Lee et al. 2006; Lin and Metters 2006; Wang and von Recum 2011). We are mainly interested in diffusion-based and affinity-based drug delivery systems because they are well suited for the PEG system we will be discussing later in this chapter. Diffusion-controlled delivery systems rely on drug release down a concentration gradient while affinity controlled systems use affinity-based interactions, such as ionic interactions, hydrophobic interactions, hydrogen bonding, or van der Waals forces to control drug release (Wang and von Recum 2011). Implementing diffusion-based drug delivery for VEGF will result in a quick release of VEGF that mimics a nonlinear Fickian release profile (Amsden 1998; Amsden and Turner 1999; Peppas, Huang et al. 2000; Arifin, Lee et al. 2006; Lin and Metters 2006; Wang and von Recum 2011). However, a system that relies only on passive diffusion will normally see the growth factor cleared away relatively quickly (Arifin, Lee et al. 2006; Lin and Metters 2006). While the burst release of VEGF is desirable, VEGF release must also be sustained for successful angiogenesis. To achieve sustained release of growth factors, an affinity-based approach is needed. A well-established affinity-based system is the heparin gel system formed by crosslinking heparin with PEG (Tae, Scatena et al. 2006). Heparin is a linear polysaccharide that reversibly binds with many proteins through mostly electrostatic interactions as well as hydrogen bonding and hydrophobic effects (Sakiyama-Elbert and Hubbell 2000; Tae,
Scatena et al. 2006; Nie, Baldwin et al. 2007; Sakiyama-Elbert 2014). Tae et al. formed these PEG-heparin gels by crosslinking the N-hydroxysuccinimidy l ester of PEG-bis-butanoic acid with hydrazide-functionalized heparin. When implanted in mice, these scaffolds demonstrated an initial burst release followed by controlled release of VEGF over three weeks that increased angiogenesis (Tae, Scatena et al. 2006). The success of their work means incorporating a similar heparin drug delivery system for VEGF in our modular microsphere-based scaffolds may be promising for vascularization.

An affinity-based drug delivery system will also be suitable for releasing S1P. However, S1P is a bioactive lipid and cannot use heparin as a delivery vehicle. Instead, it has been shown to bind lipoproteins, such as low density lipoprotein and high density lipoprotein (Murata, Sato et al. 2000; Alford, Kaneda et al. 2009). S1P has also been shown to bind globular proteins, such as albumin (Murata, Sato et al. 2000). One successful affinity-based system used BSA as the delivery vehicle for S1P (English, Welch et al. 2000; Wacker, Scott et al. 2006; Scott, Nichols et al. 2010). The affinity-based system demonstrated endothelial cells responding to a controlled release of S1P (Wacker, Scott et al. 2006; Scott, Nichols et al. 2010). Due to its success in controlled release of S1P, this delivery system was chosen to control the delivery of S1P to promote angiogenesis.

1.4 Scaffold Materials

Construction of scaffolds for tissue engineering has mainly involved the use of hydrogels due to their ability to provide a three-dimensional (3D) environment for cell growth that mimics
soft tissues. This allows limited diffusion of nutrients, oxygen, and waste through their elastic networks (Lee and Mooney 2001; Hoffman 2002; Zhu 2010). The two major types of biomaterials used to make hydrogels are natural and synthetic biomaterials. Natural biomaterials encompass all natural polymer-based materials, including polysaccharides like hyaluronic acid, dextran, and alginate chitosan, and proteins like fibrin, collagen, and gelatin (Zhu 2010). Synthetic biomaterials include synthetic polymers, such as polypeptides, PEG, poly(acrylic acid) (PAA), poly(vinyl alcohol) (PVA), and polyacrylamide (Zhu 2010). The advantages of natural materials include access to biological functions, such as biodegradation and cell adhesion (Zhu 2010). However, the use of natural polymers raises concerns of possible infection, immunogenic reactions, and mechanical stability (Nerem and Seliktar 2001; Orban, Wilson et al. 2004; Chen and Hunt 2007). Synthetic biomaterials have a number of advantages over natural biomaterials, providing adjustable mechanical properties, controllable chemical compositions, ability for photopolymerization, controllable scaffold architecture, biofunctionality, and flexible transport properties (Hoffman 2002; Drury and Mooney 2003). Due to these major advantages, synthetic biomaterials have become popular for scaffold formation.

1.4.1 Poly(ethylene glycol) as a Biomaterial

PEG is a synthetic polymer composed of ethylene oxide monomers that is sometimes referred to as poly(ethylene) oxide. Its general formula is HO-(CH₂-CH₂-O)ₙ-H, and it is commercially available in a wide range of molecular weights. Of the many synthetic biomaterials available, PEG is a popular FDA-approved hydrophilic polymer for tissue engineering due to a number of additional desirable properties including non-immunogenicity,
resistance to protein adsorption, good biocompatibility, and versatile functionalization (e.g. vinyl sulfone, amine, acrylate, thiol, azide, alkyne, and cyclooctyne) (Lee, Lee et al. 1995; Peppas, Keys et al. 1999; Alcantar, Aydil et al. 2000; Elbert and Hubbell 2001; Wacker, Scott et al. 2006; Scott, Nichols et al. 2010; Nguyen, Snyder et al. 2013). A major characteristic of PEG is its ability to form a hydration shell that extends several hundred angstroms from the PEG backbone by bonding three water molecules per each unit of PEG (Liu and Parsons 1969; Horne, Almeida et al. 1971; Kjellander and Florin 1981; Muller and Rasmussen 1991). The resistance to protein adsorption has been attributed to the hydration shell of PEG, the steric repulsion of its polymer chains, and the overall neutral charge of PEG (Jeon and Andrade 1991; Jeon, Lee et al. 1991; McPherson, Shim et al. 1997; Ostuni, Chapman et al. 2001). PEG’s high resistance to protein adsorption contributes to its non-immunogenic and biocompatibility properties. Due to these characteristics, it does not possess any biological functions that natural materials offer. In addition, PEG can be quite versatile, since it can be modified with a variety of functional groups. The wide variety of functional groups available to PEG permits the use of a number of reactions, including Michael-type addition (Huang, Michel et al. 2001), click chemistry (Nguyen, Snyder et al. 2013), enzymatic reaction (Sperinde and Griffith 1997), native chemical ligation (Hu, Su et al. 2009), irradiation of linear or branched PEG polymers (Bray and Merrill 1973), free radical polymerization (Sawhney, Pathak et al. 1993), mixed-mode polymerizations (Lee, Dalsin et al. 2002; Salinas and Anseth 2008), and others (Peppas, Keys et al. 1999; Zhu 2010). PEG derivatives can allow even more versatility through introduction of biological functions, such as degradability or delivery of biological factors (Zhu 2010). This gives PEG the same beneficial properties found in natural biomaterials, making it the ideal candidate as a biomaterial for scaffold fabrication.
1.5 Click Chemistry

Recently, click chemistry has become popular with click reactions replacing widely used crosslinking and bio-conjugation chemistries, such as free radical polymerizations, aldehydes and their Schiff base intermediates, and Michael-type additions (Hudalla and Murphy 2009). Click chemistry usually refers to the copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction, or alternatively the [3+2] azide-alkyne cycloaddition, but it can also refer to the strain-promoted [3+2] azide-alkyne cycloaddition, the Staudinger ligation, thiol-yne reactions, and other types of reactions (Kolb, Finn et al. 2001; Agard, Prescher et al. 2004; Fairbanks, Scott et al. 2009; Nwe and Brechbiel 2009). These are simple reactions that have fast kinetics, high yields, mild reaction conditions, insensitivity to oxygen or water, stereospecificity, and regiospecificity (Kolb, Finn et al. 2001; DeForest, Polizzotti et al. 2009). They can be completed under physiological conditions to produce physiologically stable products without the risk of harmful byproducts (Kolb, Finn et al. 2001; Nwe and Brechbiel 2009). Click chemistry reactions are preferred over other chemistries, which rely heavily on reaction conditions and are susceptible to hydrolysis and non-selective conjugation with biomolecule functionalities (e.g. amines) (Hudalla and Murphy 2009). Furthermore, click reactions are rapid and efficient, and the products that form are highly stable (Hudalla and Murphy 2009). Click reactions can also be called bioorthogonal because they are highly specific reactions that are biocompatible and not susceptible to non-selective conjugations (Prescher and Bertozzi 2005; McKay and Finn 2014). However, one disadvantage of the [3+2] cycloaddition reaction widely used in click chemistry is the requirement of a copper catalyst in order to perform the reaction efficiently at physiological
temperatures and environments. Copper has been shown to be toxic to bacteria and mammalian cells, which precludes the use of this reaction in the presence of cells unless the copper can be removed or neutralized (Agard, Prescher et al. 2004). Fortunately, a viable alternative to activating alkynes for catalyst-free [3+2] cycloaddition with azides can be performed using ring strain from cycloalkynes, which causes a destabilization of the ground state over the transition state to provide an accelerated reaction rate (Shea and Kim 1992; Agard, Prescher et al. 2004). Other similar alternatives that are catalyst-free include activated alkyne reactions and electron-deficient alkyne reactions (Becer, Hoogenboom et al. 2009). The advantages of click reactions make it an important consideration for PEG scaffold formation.

1.6 Scaffold Design

The success of scaffold implants for vascularization relies upon the choice of scaffold materials used, the crosslinking chemistries implemented, and especially the overall scaffold design. Poor scaffold design can easily negate the attributes of the materials and chemistries used to create the scaffold. Ideal properties of scaffolds for tissue engineering include biocompatibility, biodegradability or bioresorbability (with degradation or resorption rates that match the cellular environment), versatile surface chemistry for the addition of biological functions, mechanical properties similar to tissues at the site of implantation, a 3D structure with an interconnected pore network, and flexible fabrication process (Hutmacher 2001; Chan and Leong 2008). Of the properties listed, a particularly important design consideration for scaffold vascularization is pore size and network. It has been shown that scaffolds with pores sizes
greater than 250 µm have greater vessel ingrowth than those with smaller pore sizes (Druecke, Langer et al. 2004). However, pore size bears no significance if the pores are not interconnected to allow cell migration for vascularization. Large pore sizes in combination with pore interconnectivity are required for the success of vascularization in scaffolds (Yang, Leong et al. 2001; Karageorgiou and Kaplan 2005). The conventional methods that have been used to create porous scaffolds are phase separation (Nichols, Scott et al. 2009; Scott, Nichols et al. 2010; Smith, Segar et al. 2012; Nguyen, Snyder et al. 2013), freeze drying (Whang, Thomas et al. 1995; Whang, Tsai et al. 1998), particulate leaching (Hutmacher 2001), and gas foaming (Harris, Kim et al. 1998). These techniques for fabricating scaffolds have parameters that can be manipulated to change the size, shape, and interconnectivity of the pores, but pore organization is harder to control (Rouwkema, Rivron et al. 2008). Recently, solid free-form fabrication systems have become popular because they offer better control of the porosity and interconnectivity of pores, and the scaffolds formed tend to be well-defined instead of randomly patterned (Hutmacher, Sittinger et al. 2004; Hollister 2005). Another current fabrication technique uses dissolvable sacrificial templates that create precise patterned networks (Miller, Stevens et al. 2012; Mohanty, Larsen et al. 2015). Unlike other systems, this fabrication method produces precise networks by 3D printing the desired porous network template in a dissolvable material like carbohydrate glass or PVA and encapsulating the lattice with a biomaterial before dissolving the lattice (Miller, Stevens et al. 2012; Mohanty, Larsen et al. 2015). This system replaces pores with interconnected channels, creating ideal scaffolds for tissue engineering applications. Designing scaffolds with ideal properties has been a challenging balancing act, and until recently, creating an ideal interconnected porous network for cells was difficult to achieve.
Chapter 2

Modular Scaffolds Using Poly(ethylene glycol) Microspheres for *In Vivo* Vascularization

2.1 Abstract

Porous modular microsphere-based PEG scaffolds were created to address the need of promoting vascularization *in vivo* for cell and tissue support. Fabrication of microspheres without the use of other surfactants, organic solvents, monomers and polymers was done by taking advantage of the lower critical solution temperature (LCST) of poly(ethylene) glycol (PEG) in sodium sulfate solutions. Microspheres were formed by thermally inducing phase separation of reactive PEG derivatives in an aqueous solution to create PEG-rich domains that coarsen in size until they reach the gel point. Microspheres were then centrifuged to form scaffolds. Different types of microspheres were used to control the bioactivity and porosity of the scaffolds. Pores in the scaffolds were formed using porogenic PEG microspheres that hydrolyze within two days of scaffold formation. Introducing cell adhesion peptides allowed for cells to attach to the scaffold and delivery of sphingosine-1-phosphate (S1P) promoted cell infiltration into the scaffold. Scaffolds were implanted into mice subcutaneously and explanted after two weeks to assess vascularization. Photoacoustic microscopy and H&E staining revealed the extent of vascularization of the scaffolds. We found that PEG scaffolds delivering S1P had more vascularization than controls.
2.2 Introduction

Scaffolds are an important part of tissue engineering because they provide structural support for cells and allow for tissue development. A variety of synthetic biomaterials are used to make scaffolds, but the many useful properties of poly(ethylene) glycol (PEG) hydrogels make them promising for use as scaffolds in tissue engineering (Scott, Nichols et al. 2010). Scaffolds made from PEG have many desirable properties including biocompatibility, resistance to protein adsorption, the ability to impart biological functions, and the means for drug delivery (Jeon and Andrade 1991; Jeon, Lee et al. 1991; Lee, Park et al. 1991; Alcantar, Aydil et al. 2000; Richardson, Peters et al. 2001; DeLong, Moon et al. 2005; Lutolf and Hubbell 2005). Porous PEG hydrogels are optimal for tissue-engineered scaffolds, since their porosity allows for cell function and survivability. (Lutolf and Hubbell 2005; Varghese and Elisseeff 2006; Nguyen, Snyder et al. 2013). Porous scaffolds can be created using several techniques, such as incorporating nontoxic porogens or creating macro networks from dissolvable materials (Asnaghi, Giglio et al. 1995; Shapiro and Cohen 1997; Lévesque, Lim et al. 2005; Stachowiak, Bershteyn et al. 2005; Ford, Bertram et al. 2006; Sannino, Netti et al. 2006; Bryant, Cuy et al. 2007; Osathanon, Linnes et al. 2008; Miller, Stevens et al. 2012). Scaffolds can recruit cells and promote attachment by delivering bioactive molecules, such as sphingosine-1-phosphate (S1P), and imparting biological functions, such as cell adhesion. (Richardson, Peters et al. 2001; DeLong, Moon et al. 2005; Lutolf and Hubbell 2005; Wacker, Scott et al. 2006; Scott, Nichols et al. 2010). Incorporating these properties into tissue-engineered scaffolds is one method for promoting cell migration and proliferation that will result in scaffold vascularization.
An effective method for engineering the properties of a scaffold is to modularly assemble scaffolds with different parts that introduce different properties. Scaffolds made using the modular approach consist of various types of microparticles that have different functionalities or characteristics to tailor the scaffold for specific purposes (McGuigan and Sefton 2006; Yeh, Ling et al. 2006; Rivest, Morrison et al. 2007; Du, Lo et al. 2008; Pautot, Wyart et al. 2008; Serban and Prestwich 2008; Scott, Nichols et al. 2010). A technique that has been shown to successfully form scaffolds with properties tailored for cell survival, attachment, and migration is the formation of PEG functional microspheres that can be crosslinked together to form modular scaffolds (Nichols, Scott et al. 2009; Scott, Nichols et al. 2010). This method creates PEG microspheres without a surfactant by exploiting the lower critical solution temperature (LCST) of PEG derivatives with kosmotropic salts to form PEG-rich domains that coarsen and crosslink into microspheres (Bailey and Callard 1959; Bae, Lambert et al. 1991; Nichols, Scott et al. 2009; Scott, Nichols et al. 2010; Flake, Nguyen et al. 2011). These microspheres can then crosslink together to form a modular scaffold.

Modular scaffolds made from PEG microspheres have been created as previously described (Nichols, Scott et al. 2009; Scott, Nichols et al. 2010). Three types of microspheres were used to create the scaffolds (Figure 2.1). There were structural microspheres that provided support and cell adhesion, microspheres that delivered S1P, and porogenic microspheres that contained hydrolyzable ester bonds. Unreacted reactive groups on the surface of the microspheres were further crosslinked with BSA to fabricate the scaffolds (Figure 2.1). These modular microsphere scaffolds that were shown to be porous and capable of supporting endothelial cell infiltration (Scott, Nichols et al. 2010) were implanted into mice to assess their in vivo vascularization.
2.3 Materials and Methods

2.3.1 PEG₈-Vinyl Sulfone Synthesis

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich. The synthesis procedure was adapted from a procedure as previously described (Morpurgo, Veronese et al. 1996; Scott, Nichols et al. 2008). Eight-arm PEG-mesylate (PEG₈-mesylate; mol wt 10,000) was first synthesized from eight-arm PEG-OH (PEG₈-OH; mol wt 10,000; Creative PEGWorks) by mesylating the alcohol group on PEG₈-OH with mesyl chloride. This was done by dissolving PEG₈-OH in toluene at 10% (w/v), drying the solution by azeotropic distillation, and cooling the solution to room temperature. Dichloromethane (DCM) was added to 20% (v/v) before putting the solution on ice. Four equivalents of triethylamine and four equivalents of methanesulfonyl chloride were added while on ice, and the reaction continued overnight under constant stirring.
and nitrogen flow. After removing the salt byproduct, excess DCM was removed by using the rotovap, and the PEG$_8$-mesylate was precipitated out using cold diethyl ether. The product was dried under vacuum overnight to remove any remaining diethyl ether. The PEG$_8$-mesylate was then converted to PEG$_8$-hydroxyethyl sulfide. To do this, the PEG$_8$-mesylate was dissolved in deionized water at 10% (w/v) and four equivalents of 1M sodium hydroxide (NaOH) and four equivalents of β-mercaptoethanol were added. The reaction was then performed at reflux for three hours. Then the product was cooled on ice and extracted twice with DCM over anhydrous sodium sulfate (Na$_2$SO$_4$). After removing the salt byproduct, excess DCM was removed by using the rotovap, and the PEG$_8$-hydroxyethyl sulfide was precipitated out using cold diethyl ether. The product was dried under vacuum overnight to remove any remaining diethyl ether. The PEG$_8$-hydroxyethyl sulfide was then dissolved in 0.123 M sodium tungstate in distilled water at 25% (w/v). Two equivalents of 30% hydrogen peroxide were added to the solution while on ice, and the reaction continued overnight under constant stirring and nitrogen flow. Then the product was extracted twice with DCM over anhydrous Na$_2$SO$_4$. After removing the salt byproduct, excess DCM was removed by using the rotovap, and the PEG$_8$-hydroxyethyl sulfone was precipitated out using cold diethyl ether. The product was dried under vacuum overnight to remove any remaining diethyl ether. The PEG$_8$-hydroxyethyl sulfone was then dissolved in toluene at 10% (w/v), dried through azeotropic distillation, and cooled to room temperature. DCM was added to 20% (v/v) before putting the solution on ice. Three equivalents of triethylamine and 1.5 equivalents of methanesulfonyl chloride were added while on ice, and the reaction continued overnight under constant stirring and nitrogen flow. Another three equivalents of triethylamine and 1.5 equivalents of methanesulfonyl chloride were added while on ice, and the reaction continued again overnight under constant stirring and nitrogen flow.
After removing the salt byproduct, excess DCM was removed by using the rotovap, and the PEG8-vinyl sulfone was precipitated out using cold diethyl ether. The product was dried under vacuum overnight to remove any remaining diethyl ether. Again the product was extracted twice with DCM over anhydrous Na₂SO₄. After removing the salt byproduct, excess DCM was removed by using the rotovap, and the PEG8-vinyl sulfone was precipitated out using cold diethyl ether. The product was dried under vacuum overnight to remove any remaining diethyl ether. ¹H NMR (300 MHz, CDCl₃, δ): 3.6 (PEG backbone), 6.35 (d, 2H, =CH₂), 6.8 (m, 1H, -SO₂CH-). NMR of the product confirmed vinyl sulfone features at 6.35 ppm and 6.8 ppm.

2.3.2 PEG₈-Amine Synthesis

The synthesis procedure was adapted from a procedure as previously described (Elbert and Hubbell 2001). PEG₈-mesylate was synthesized as described earlier. Eight-arm PEG-amine (PEG₈-amine; mol wt 10,000) was then synthesized from PEG₈-mesylate by an amination reaction with ammonium hydroxide. PEG₈-mesylate was first dissolved it in 400 mL of 28-30% ammonium hydroxide. The PEG₈-mesylate was constantly stirred in a sealed bottle for 3-4 days. Afterwards, the bottle was uncapped to allow the ammonia to evaporate while still under constant stirring for 3-5 days. To collect the product, the pH of the solution was raised to 13 with 1 M NaOH, and then extracted with DCM over anhydrous Na₂SO₄. A standard DCM extraction procedure was done to extract the PEG₈-amine into the DCM. After three extractions, the Na₂SO₄ was filtered out and the combined DCM phase was rotovapped, diethyl ether precipitated, and dried as was done for the PEG₈-mesylate. ¹H NMR (300 MHz, CDCl₃, δ): 3.6
(PEG backbone), 2.9 (t, 16H, -CH₂-). NMR of the product confirmed an amine end group by the presence of a triplet at 2.9 ppm.

2.3.3 PEG₈-Acrylate Synthesis

The synthesis procedure was adapted from a procedure as previously described (Elbert and Hubbell 2001). Eight-arm PEG-acrylate (PEG₈-acrylate; mol wt 10,000) was synthesized from PEG₈-OH. This was done by dissolving PEG₈-OH in toluene at 10% (w/v), drying the solution by azeotropic distillation, and cooling the solution to room temperature. DCM was added to 20% (v/v) before putting the solution on ice. 1.5 equivalents of triethylamine and 1.5 equivalents of acryloyl chloride were added while on ice, and the reaction continued overnight under constant stirring and nitrogen flow. After removing the salt byproduct, excess DCM was removed by using the rotovap, and the PEG₈-acrylate was precipitated out using cold diethyl ether. The product was dried under vacuum overnight to remove any remaining diethyl ether. The product was then dissolved in deionized water at 10% (w/v) and sodium chloride was added until the solution reached 5% (w/v). The pH was adjusted to pH 6 with NaOH, and the product was extracted three times with DCM over anhydrous Na₂SO₄. After removing the any excess salts and byproducts, excess DCM was removed by using the rotovap, and the PEG₈-acrylate was precipitated out using cold diethyl ether. The product was dried under vacuum overnight to remove any remaining diethyl ether. ¹H NMR (300 MHz, CDCl₃, δ): 3.6 (PEG backbone), 4.3 (t, 16H, -CH₂-COO-), 5.8 (dd, 8H, -COO-CH₂=CH). NMR of the product confirmed an acrylate end group by the presence of a triplet at 4.3 ppm and a doublet of doublets at 5.8 ppm.
2.3.4 Microgel Formation and Characterization with Dynamic Light Scattering

The synthesis procedure was adapted from a procedure as previously described (Scott, Nichols et al. 2008; Scott, Nichols et al. 2010). Solutions of PEG₈-vinyl sulfone, PEG₈-amine, PEG₈-acyrlate and bovine serum albumin (BSA) were prepared at 20% (w/v) in phosphate buffered saline (PBS; Pierce; pH 7.4) and sterile filtered with 0.22 µm syringe tip filters (Millipore). Microgel solutions were formed to overcome the slightly higher reactivity of amines with vinyl sulfones than acrylates (Friedman, Cavins et al. 1965; Scott, Nichols et al. 2010). Through Michael-type conjugate addition reactions, PEG₈-vinyl sulfone/BSA and PEG₈-vinyl sulfone/PEG₈-amine microgel solutions were formed by mixing PEG₈-vinyl sulfone with either BSA or PEG₈-amine solutions at ratios of 1:1 of vinyl sulfone to amine groups. It was assumed that 8 moles of accessible functional groups per mole of PEG₈-vinyl sulfone or PEG₈-amine were available, and approximately 36 lysines were sterically accessible for reaction on BSA (Wacker, Scott et al. 2006). PEG₈-amine/PEG₈-acrylate microgel solutions were formed by mixing the PEG₈-amine and PEG₈-acrylate solutions at ratios of 1:1 of amine to acrylate groups. The microgel solutions were rotated at 40 RPM at 37°C until the mean effective hydrodynamic diameters ($d_{PCS}$) reached an average of 100 nm as determined by dynamic light scattering/photon correlation spectroscopy (DLS/PCS; 90Plus Particle Size Analyzer, Brookhaven Instruments, Holtsville, NY). The measurement was performed at a scattering angle of 90° and wavelength of 658 nm. Brookhaven Instruments Particle Sizing Software (version 2.34, Brookhaven Instruments) was used to calculate the $d_{PCS}$ and generate statistical analysis of the results.
2.3.5 Microsphere Fabrication

The fabrication process was adapted from a procedure as previously described (Scott, Nichols et al. 2008; Scott, Nichols et al. 2010). The microgel solution of PEG₈-vinyl sulfone/PEG₈-amine was diluted to 2% (w/v) at room temperature with PBS and 1.5 M Na₂SO₄ in PBS to reach a final Na₂SO₄ concentration of 0.6 M and volume of 250 µL. The microgel solution of PEG₈-vinyl sulfone/BSA was diluted to 2% (w/v) at room temperature with PBS and 1.5 M Na₂SO₄ in PBS to reach a final Na₂SO₄ concentration of 0.65 M and volume of 250 µL. The microgel solution of PEG₈-amine/PEG₈-acrylate was diluted to 2% (w/v) at room temperature with PBS and 1.5 M Na₂SO₄ in PBS to reach a final Na₂SO₄ concentration of 0.45 M and volume of 250 µL. Unless otherwise noted, the concentration of Na₂SO₄ was chosen to lower the LCST of PEG such that the PEG phase separated above room temperature but below the reaction temperature. The solutions were then heated above the cloud point at 37°C for PEG₈-vinyl sulfone/BSA and PEG₈-vinyl sulfone/PEG₈-amine, and the solutions were heated above the cloud point at 95°C for PEG₈-amine/PEG₈-acrylate. Unless otherwise noted, the reaction was performed long enough such that the microspheres formed but aggregation was kept to a minimum. To remove the Na₂SO₄, the microspheres were then buffer exchanged into PBS by diluting them 3:1 with PBS, triturating, and centrifuging at 14,100 x g for 2 minutes. This process was repeated two more times.
2.3.6 S1P and RGD Incorporation into Microsphere Fabrication

This process was adapted from a procedure as previously described (Scott, Nichols et al. 2008; Scott, Nichols et al. 2010). To deliver S1P (BIOMOL International), dried S1P was added to 20% (w/v) BSA in PBS at 1 nmol S1P/mg BSA and rotated at 37°C until S1P was solubilized. This solution was then added to PEG₈-vinyl sulfone to form microgel solutions as previously described. The PEG₈-vinyl sulfone/BSA/S1P microgels were then used to form microspheres in the same manner as previously described for PEG₈-vinyl sulfone/BSA microspheres.

The addition of the cell adhesion peptide was done by mixing the pre-reacted solution of PEG₈-vinyl sulfone/PEG₈-amine (2:1 vinyl sulfone:amine, $d_{PCS} \approx 50$ nm, 20% (w/v) PEG) with RGD peptide (Seq: GCGYGrRGDSPG; GenScript USA Inc.) at an RGD concentration of 5.5 mM for 30 min at 37°C. This formed PEG₈-vinyl sulfone/PEG₈-amine/RGD microgels. The PEG₈-vinyl sulfone/PEG₈-amine/RGD microgels were then used to form microspheres in the same manner as previously described for PEG₈-vinyl sulfone/PEG₈-amine.

2.3.7 Scaffold Formation for Implantation

Two sets of scaffolds were made for implantation. The control set comprised of PEG₈-vinyl sulfone/PEG₈-amine/RGD microspheres, PEG₈-vinyl sulfone/BSA microspheres, and PEG₈-amine/PEG₈-acrylate microspheres in a ratio of 1:1:1. Scaffolds delivering S1P contained a mixture of PEG₈-vinyl sulfone/PEG₈-amine/RGD microspheres, PEG₈-vinyl sulfone/BSA/S1P microspheres, and PEG₈-amine/PEG₈-acrylate microspheres in a ratio of 1:1:1. The implantation scheme was a modification of a method developed by Chen et al. (Chen, Aledia et al. 2009). For
each scaffold type, the microspheres were mixed together with 2% (w/v) BSA in PBS and placed in polydimethylsiloxane (PDMS; Dow Corning) chambers that had a height of 8 mm, an inner diameter of 8 mm, and an outer diameter of 10 mm. These chambers simplified scaffold implantation and limited the infiltration of blood vessels to one side of the scaffold. The microspheres were centrifuged at 1000 x g for 10 min in the PDMS chambers. Excess BSA and PBS were removed and the PDMS chambers were trimmed until they were level with the scaffold.

2.3.8 Scaffold Implantation and Explantation in Mice

Outbred mice (Hsd:ICR; Harlan) were used for the scaffold experiments. The mice were anesthetized, shaved, and cleaned prior to surgery. A 1.5 cm incision along the middle section of the mouse’s back was made. Following the incision, subcutaneous pockets were formed underneath the skin with iris scissors at the left flank and right flank of the mouse. Scaffolds without S1P were placed in the left flank, and scaffolds with S1P were placed in the right flank of the mouse. PDMS chambers were oriented upright with scaffolds facing the skin. The incision was then sutured up with Vicryl (Ethicon) suture and sealed with VetBond (3M). After 14 days, the mice were sacrificed, and the scaffolds were removed and fixed in 3.7% (w/v) formaldehyde solution for at least 24 hours.
2.3.9 Photoacoustic Microscopy

Optical-resolution photoacoustic microscopy was performed on the fixed samples according to a procedure that has been previously reported (Geng, Xueding et al. 2004; Xu and Wang 2006; Rao, Li et al. 2010). In general, nonionizing waves directed at the scaffold sample were used to excite wideband ultrasound waves that can be detected by ultrasonic transducers to form images (Xu and Wang 2006; Rao, Li et al. 2010). Photoacoustic microscopy revealed any blood vessels that may have entered or have been recruited toward the scaffold.

2.3.10 Hematoxylin and Eosin (H&E) Staining and Sectioning

Fixed scaffolds were sent to the AMP Core Lab at Washington University School of Medicine’s Department of Pathology & Immunology for H&E staining and sectioning. Samples were then taken to the Hope Center at Washington University School of Medicine where images were captured using a NanoZoomer (Hamamatsu).

2.4 Results and Discussion

2.4.1 Microsphere and Scaffold Formation

Following the procedure that was previously demonstrated to successfully form microspheres from the reaction of PEG derivatives (Scott, Nichols et al. 2008; Scott, Nichols et al. 2010), we were able to form PEG₈-vinyl sulfone/PEG₈-amine/RGD, PEG₈-amine/PEG₈-
acrylate, PEG₈-vinyl sulfone/BSA/S1P, and PEG₈-vinyl sulfone/BSA microspheres. PEG₈-vinyl sulfone/PEG₈-amine/RGD microspheres served as structural microspheres and provided adhesion peptides for the cells. PEG₈-amine/PEG₈-acrylate microspheres added porosity to the scaffolds. These porogenic microspheres degraded after two days once their bonds were hydrolyzed. PEG₈-vinyl sulfone/BSA/S1P microspheres also served as structural microspheres and as a vehicle for delivery of S1P. A set of scaffolds that served as the control group was created by suspending PEG₈-vinyl sulfone/PEG₈-amine/ RGD, PEG₈-amine/PEG₈-acrylate, and PEG₈-vinyl sulfone/BSA microspheres in 2% BSA in PBS and centrifuging them together at 1000 x g for 10 min. The scaffolds that delivered S1P were made by suspending PEG₈-vinyl sulfone/PEG₈-amine/ RGD, PEG₈-amine/PEG₈-acrylate, and PEG₈-vinyl sulfone/BSA/S1P microspheres in with 2% BSA in PBS and centrifuging them together at 1000 x g for 10 min. It had been previously demonstrated that microspheres needed to be crosslinked in 2% fetal bovine serum (FBS) to form adequate scaffolds because the serum proteins served to crosslink the microspheres through the reaction of the amines on the proteins with the vinyl sulfone groups on the microspheres (Scott, Nichols et al. 2008; Scott, Nichols et al. 2010). We replaced 2% FBS with 2% BSA in order to reduce the immunological response from the mice. The microspheres successfully crosslinked with 2% BSA to form scaffolds similar to ones that crosslinked with 2% FBS. The scaffolds were formed in PDMS chambers, so that they would be ready for implantation following fabrication.
2.4.2 Implantation of Scaffolds into Mice

After the scaffolds had been formed, they were subcutaneously implanted into mice. The procedure for implanting the scaffolds is illustrated in Figure 2.2. The controls without S1P were placed subcutaneously on the left flank of the mice, and the scaffolds delivering S1P were placed subcutaneously on the right flank of the mice. The scaffolds were placed in PDMS chambers to simplify the implantation process, limit the infiltration of blood vessels to one side of the scaffold, preserve the structural integrity of the scaffold, and simplify explantation of the scaffold. Scaffolds placed within the mice without PDMS chambers tend to shift back and forth as the mice move around, which can damage the scaffolds and complicate their explantation. The scaffolds were explanted after 14 days and remained intact.
Figure 2.2. Schematic of scaffold and illustration of implantation procedure. Once the scaffolds have been prepared, the mice were cleaned and an incision was made on their backs. Subcutaneous pockets were formed underneath the skin and scaffolds delivering S1P were placed on the right flank while control scaffolds were placed on the left flank of the mice. The incision was closed following the procedure and the mice were monitored for 14 days until the scaffolds were explanted.

2.4.3 Scaffold Explantation and Analysis

After 14 days, the scaffolds in their PDMS chambers were explanted from the mice, fixed in 3.7% formaldehyde solution for 24 hours, and analyzed by H&E staining or photoacoustic microscopy. Prior to fixing the scaffolds, macroscopic images of the scaffold implants were taken to demonstrate the extent of vascular ingrowth into the scaffolds (Figure 2.3). Vascular ingrowth into scaffolds was observed for both the control scaffolds and scaffolds delivering S1P,
but there was observably more ingrowth in scaffolds with S1P. Half of the samples were prepared for photoacoustic microscopy, and the other half were prepared for H&E staining and sectioning. The samples prepared for optical-resolution photoacoustic microscopy were excited with nonionizing waves in order to excite wideband ultrasound waves that were then detected by ultrasonic transducers to form images (Geng, Xueding et al. 2004; Xu and Wang 2006; Rao, Li et al. 2010). Photoacoustic microscopy revealed that scaffolds with S1P recruited more blood vessels than those without S1P (Figure 2.4). Images taken of scaffolds after H&E staining and sectioning also indicated that scaffolds delivering S1P had a higher prevalence of vascularization than those without S1P (Figure 2.5 and Figure 2.6). Both techniques revealed qualitatively higher recruitment of blood vessels into the scaffolds that delivered S1P than the control scaffolds.

Figure 2.3. Pictures of scaffold implants. The scaffold on the left released S1P while scaffold on the right had no S1P. There is an observable difference in vascularization between the two scaffolds. More blood vessels can be seen in the scaffold that delivered S1P.
Figure 2.4. Photoacoustic microscopy images of scaffold implants. The scaffold on the left released S1P while scaffold on the right had no S1P. There is a qualitative increase in blood vessels being recruited to the scaffold delivering S1P than the scaffold without S1P.
Figure 2.5. Histology image of PEG scaffold without S1P after implantation. The scaffold elicited an immunological response from the mouse that can be seen by the cells stained in blue bordering the scaffold. Parts of the scaffold also suffered retraction or degradation.
Figure 2.6. Histology image of PEG scaffold with S1P after implantation. The scaffold elicited an immunological response from the mouse that can be seen by the cells stained in blue bordering the scaffold. Parts of the scaffold also suffered retraction or degradation. This scaffold also has areas marked with blood vessels and their supporting network of fibroblasts that the control scaffold lacked.

The H&E images also revealed a large amount of foreign body response from the mice to both types of scaffolds. This is indicated by the cells stained in blue that either surrounded or infiltrated the scaffolds (Figure 2.5 and Figure 2.6). The images also show parts of the scaffolds experienced possible retraction or degradation that was likely due to the foreign body response. The use of BSA to form the microspheres and scaffolds was the likely cause of the immunological response from the mice. Foreign body response and inflammation has been
shown to occur with implants (Anderson 1988), and more importantly inflammation has been demonstrated to induce angiogenesis (Imhof and Aurrand-Lions 2006; Granger and Senchenkova 2010). This explains the vascularization observed with both types of scaffolds. While angiogenesis is welcomed, inflammation can lead to destruction or diseases of the surrounding tissue (Imhof and Aurrand-Lions 2006). Ideally, the BSA would be removed in order to minimize the immunological response, but removing BSA will prevent forming some of the structural microspheres required to make the scaffolds. Furthermore, BSA provided an effective means of highly crosslinking the microspheres and forming scaffolds that were strong enough to be implanted into mice. A new system would be needed to reduce the immunological response and create scaffolds strong enough for implantation.

2.5 Conclusions

We demonstrated that microsphere-based PEG scaffolds could be successfully implanted in mice and deliver S1P for the promotion of vascularization. Prior to this work, microsphere-based PEG scaffolds were shown to be highly biocompatible with endothelial cells, and they were shown to successfully introduce bioactivity in the form of RGD peptide conjugation and S1P delivery in vitro (Nichols, Scott et al. 2009; Scott, Nichols et al. 2010). These promising in vitro studies of the scaffolds had mixed in vivo results. Scaffolds delivering S1P recruited more blood vessels than those without any S1P delivery. However, H&E staining revealed a significant amount of foreign body response to the scaffolds. The BSA used to fabricate the microspheres and scaffolds was the cause of the immunological response observed. Since BSA
has a pivotal role in fabricating the microspheres and scaffolds, a different approach will be required to have micropshere-based PEG scaffolds that do not invoke a large foreign body response. We anticipate that the modular nature of these microsphere-based PEG scaffolds will present a solution that builds upon the groundwork of this study.
Chapter 3

Clickable Poly(ethylene glycol) Microsphere-Based Cell Scaffolds

3.1 Abstract

Clickable poly(ethylene glycol) (PEG) derivatives are used with two sequential aqueous two-phase systems to produce microsphere-based scaffolds for cell encapsulation. In the first step, sodium sulfate causes phase separation of the clickable PEG precursors and is followed by rapid gelation to form microspheres in the absence of organic solvent or surfactant. The microspheres are washed and then deswollen in dextran solutions in the presence of cells, producing tightly packed scaffolds that can be easily handled while also maintaining porosity. Endothelial cells included during microsphere scaffold formation show high viability. The clickable PEG microsphere-based cell scaffolds open up new avenues for manipulating scaffold architecture as compared to simple bulk hydrogels.

1 Chapter 3 has been adapted from the following manuscript: Nguyen PK, Snyder CG, Shields JD, Smith AW, Elbert DL. Clickable Poly(ethylene glycol)-Microsphere-Based Cell Scaffolds. Macromol Chem Phys. 2013 Apr 25;214(8):948-956.


3.2 Introduction

Polymeric materials are promising as scaffolds for tissue reconstruction and drug delivery systems for the therapeutic release of bioactive molecules (Langer, Vacanti et al. 1995; Griffith 2000; Hoffman 2002). Poly(ethylene glycol) (PEG) is a widely used hydrophilic polymer that resists non-specific biological interactions (Lee, Lee et al. 1995; Peppas, Keys et al. 1999; Alcantar, Aydil et al. 2000), yet is readily modified with proteins and peptides for enzymatic biodegradation and cell adhesion (West and Hubbell 1999; Lutolf and Hubbell 2005; Zhu 2010). While PEG hydrogels have been demonstrated to be useful in a number of applications, modular or self-assembling hydrogel-based scaffolds promise to facilitate the generation of complex architectures that better mimic the organization of natural tissues (Lutolf and Hubbell 2005; Elbert 2011). PEG scaffolds and hydrogels have been crosslinked using a wide variety of chemical reactions including free radical polymerization (Sawhney, Pathak et al. 1993), Michael-type addition (Huang, Michel et al. 2001), enzymatic reaction (Sperinde and Griffith 1997), irradiation of linear or branched PEG polymers (Bray and Merrill 1973), mixed-mode polymerizations (Lee, Dalsin et al. 2002; Salinas and Anseth 2008), native chemical ligation (Hu, Su et al. 2009), and other strategies (Peppas, Keys et al. 1999; Zhu 2010). Click reactions are defined as bioorthogonal reactions and include reactions such as the Huisgen 1,3 dipolar cycloaddition between azides and alkynes, thiol-ene/ynel photoadditions, and Staudinger ligation (ten Brummelhuis, Diehl et al. 2008; Iha, Wooley et al. 2009; Nwe and Brechbiel 2009; Hoyle, Lowe et al. 2010; Park, Kloxin et al. 2010). Taking advantage of the rapid reaction kinetics of copper(I)-catalyzed azide-alkyne cycloaddition, crosslinked hydrogel networks have been produced and tailored for various applications (Malkoch, Vestberg et al. 2006; Ossipov and
Crosslinked polymer networks have also been fabricated and modified for several applications using thiol-ene/yne photoadditions (Polizzotti, Fairbanks et al. 2008; Fairbanks, Scott et al. 2009; Impellitteri, Toepke et al. 2012). Copper-free azide-alkyne cycloadditions have recently attracted attention for materials fabrication, as these reactions have high conversions, fast kinetics, insensitivity to oxygen and water, stereospecificity, regiospecificity, and mild reaction conditions (Johnson, Baskin et al. 2008; Clark and Kiser 2009; DeForest, Polizzotti et al. 2009; DeForest, Sims et al. 2010). Additionally, the reactions can be performed under physiological conditions with little risk of non-specific reactions with molecules found in cells and tissues (Nwe and Brechbiel 2009). Copper-free cycloadditions have been designed to allow incorporation of protease-sensitive peptides to enable enzymatic biodegradation (DeForest, Polizzotti et al. 2009; Fairbanks, Schwartz et al. 2009; DeForest, Sims et al. 2010; van Dijk, van Nostrum et al. 2010) and attachment of cell adhesion peptides through photoaddition (Polizzotti, Fairbanks et al. 2008; DeForest, Polizzotti et al. 2009; Fairbanks, Schwartz et al. 2009; DeForest, Sims et al. 2010). Due to the exceedingly slow reaction kinetics of alkynes with thiols, amines, alcohols, etc., PEG scaffolds formed using click reactions may be useful for cell transplantation and drug delivery.

We previously developed techniques for the fabrication of PEG microspheres by phase inversion polymerization (Nichols, Scott et al. 2009; Scott, Nichols et al. 2010; Elbert 2011). By lowering the lower critical solution temperature (LCST) of PEG with kosmotropic salts, reactive PEG derivatives underwent a thermally induced phase separation. Spherical PEG-rich domains coalesced and rapidly increased in size due to the absence of surfactants or stirring. However,
coalescence was halted when the spherical domains reached the gel point (Nichols, Scott et al.
2009). We previously found that the mean size of the microspheres could be controlled quite
precisely by altering the kinetics of the reaction, such that the gel point was reached at different
times following phase separation (Nichols, Scott et al. 2009). We sought to demonstrate the
phase inversion polymerization technique could be adapted to azide-alkyne cycloadditions.

3.3 Materials and Methods

3.3.1 PEG₄-Azide Synthesis

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich. Four-arm
PEG-mesylate (PEG₄-mesylate; mol wt 10,000) was first synthesized from four-arm PEG-OH
(PEG₄-OH; mol wt 10,000; Creative PEGWorks) by mesylating the alcohol group on PEG₄-OH
with mesyl chloride. This was done by dissolving PEG₄-OH in toluene at 10% (w/v), drying the
solution by azeotropic distillation, and cooling the solution to room temperature.
Dichloromethane (DCM) was added at 20% (v/v) before putting the solution on ice. Four
equivalents of triethylamine and four equivalents of methanesulfonyl chloride were added while
on ice, and the reaction continued overnight under constant stirring and nitrogen flow. After
removing the salt byproduct, excess DCM was removed by using the rotovap, and the PEG₄-
mesylate was precipitated out using cold diethyl ether. The product was dried under vacuum
overnight to remove any remaining diethyl ether. The next step was the nucleophilic azidation of
the mesylate group with sodium azide. Three equivalents of sodium azide were dissolved in
dimethylformamide (DMF). PEG₄-mesylate was then dissolved in the DMF mixture at 10%
(w/v) and put under nitrogen and constant stirring in a hot water bath at 60°C. The reaction was run overnight. The following day required the filtration of excess salt followed by rotovapping, diethyl ether precipitation, and drying as was done for the PEG₄-mesylate. The product was dissolved in a basic water solution with a pH between 9 and 12, and then extracted with DCM over anhydrous sodium sulfate (Na₂SO₄). A standard extraction procedure was done to extract the product into DCM. After three extractions, the Na₂SO₄ was filtered out and the process of rotovapping, diethyl ether precipitation, and drying was done as before. ¹H NMR (300 MHz, CDCl₃, δ): 3.6 (PEG backbone), 3.0 (s, 3H, -SO₂CH₃), 4.3 (t, 2H, -CH₂OSO₂⁻). NMR of the product confirmed that no mesylate features remained at 3.0 ppm and 4.3 ppm (Figure 3.1).
Figure 3.1. NMR of PEG₄-azide. PEG₄-azide was synthesized using a two-step process. The PEG backbone is located at 3.6 ppm. The conversion of mesylate to azide is shown by the absence of signals at 3.0 ppm and 4.3 ppm.

3.3.2 PEG₄-Alkyne Synthesis

PEG₄-mesylate was synthesized as described earlier. Four-arm PEG-amine (PEG₄-amine; mol wt 10,000) was then synthesized from PEG₄-mesylate by an amination reaction with ammonium hydroxide. PEG₄-mesylate was first dissolved it in 400 mL of 28-30% ammonium hydroxide. The PEG₄-mesylate needed to be constantly stirred in a sealed bottle for 3-4 days.
Afterwards, the bottle was uncapped to allow the ammonia to evaporate while still under constant stirring, which took 3-5 days. To collect the product, the pH of the solution was raised to 13 with sodium hydroxide, and then extracted with DCM over anhydrous Na₂SO₄. A standard DCM extraction procedure was done to extract the PEG₄-amine into the DCM. After three extractions, the Na₂SO₄ was filtered out and the combined DCM phase was rotovapped, diethyl ether precipitated, and dried as was done for the PEG₄-mesylate. The final step was PEG₄-alkyne synthesis from PEG₄-amine. PEG₄-amine was dissolved in DCM in a beaker, and 1.5 equivalents of diisopropylcarbodiimide (DIPCDI) was added to a separate spherical flask with DCM while on ice and under nitrogen flow and constant stirring. Next, 1.5 equivalents of hydroxybenzotriazole (HOBT) and 1.5 equivalents of propiolic acid were added to the mixture in the flask and allowed to stir for 10 minutes. While waiting, three equivalents of N,N-diisopropylethylamine (DIPEA) were added to the dissolved PEG₄-amine. Finally, this mixture was slowly added to the spherical flask, and the reaction was allowed to go for 24 hours in the ice bath, under constant stirring and nitrogen gas. Following that process, the urea precipitate was filtered out, and rotovapping, diethyl ether precipitation, and drying were performed. The product was then dissolved in deionized water and underwent the same extraction procedure that was done for the PEG₄-amine. Further rotovapping, diethyl ether precipitation, and drying were done. ¹H NMR (300 MHz, CDCl₃, δ): 3.6 (PEG backbone), 2.1 (t, H, ≡CH). NMR of the product confirmed the synthesis of PEG₄-alkyne with the presence of a peak at 2.1 ppm (Figure 3.2).
Figure 3.2. NMR of PEG₄-alkyne. PEG₄-azide was synthesized using a three-step process. The PEG backbone is located at 3.6 ppm. The conversion of amines to alkynes is shown by the signal at 2.1 ppm.

3.3.3 PEG₄-Cyclooctyne Synthesis

The four-arm PEG-cyclooctyne (PEG₄-cyclooctyne) synthesis was identical to the PEG₄-alkyne synthesis with the only difference being the use of an aza-dibenzocyclooctyne with a pendant carboxylic acid (DBCO-acid; Click Chemistry Tools) instead of propiolic acid. $^1$H NMR (300 MHz, CDCl₃, δ): 3.6 (PEG backbone), 5.1 (d, 2H, -CH₂-). NMR of the product
confirmed the synthesis of PEG₄-cyclooctyne with the presence of a doublet at 5.1 ppm (Figure 3.3).

**Figure 3.3.** NMR of PEG₄-cyclooctyne. PEG₄-cyclooctyne was synthesized using a three-step process. The PEG backbone is located at 3.6 ppm. The conversion of amines to cyclooctynes is shown by the signal at 5.1 ppm.
3.3.4 Preparation of Stock Solutions of PEG₄-Azide, PEG₄-Alkyne, PEG₄-Cyclooctyne, and PEG-Dithiol

All PEG derivatives were synthesized as described earlier with the exception of linear PEG-thiol (PEG-dithiol; mol wt 3400; Creative PEGWorks). Stock solutions of each PEG derivative were prepared at 20% (w/v) in phosphate buffered saline (PBS; Pierce; pH 7.4).

3.3.5 PEG₄-Azide/PEG₄-Alkyne Hydrogel Synthesis

PEG₄-azide/PEG₄-alkyne hydrogels were formed at room temperature or 37°C by combining stock solutions of PEG₄-azide and PEG₄-alkyne in a ratio of 1:1 with PBS, five equivalents of sodium L-ascorbate and 0.5 equivalents copper sulfate (Cu(II)SO₄). Equivalents were based on moles of azide. After at least 2 minutes, 0.1 M ethylenediaminetetraacetic acid (EDTA) in PBS was added to the sample to chelate the copper. Excess copper was removed by washing two more times with 0.1 M EDTA and three times with PBS. The final PEG percentage was 10% (w/v).

3.3.6 PEG₄-Azide/PEG₄-Cyclooctyne Hydrogel Synthesis

PEG₄-azide/PEG₄-cyclooctyne hydrogels were formed at room temperature or 37°C by combining stock solutions of PEG₄-azide and PEG₄-cyclooctyne in a ratio of 1:1 with PBS. The final PEG percentage was 10% (w/v).
3.3.7 PEG₄-Azide/PEG₄-Alkyne Microsphere Fabrication

Stock solutions of PEG₄-azide and PEG₄-alkyne were combined with PBS, Na₂SO₄, sodium L-ascorbate, and Cu(II)SO₄. The final solution was in PBS and Na₂SO₄ with five equivalents of sodium L-ascorbate and 0.5 equivalents Cu(II)SO₄ (equivalents based on moles of azide). Unless otherwise noted, the concentration of Na₂SO₄ was chosen to lower the LCST of PEG such that the PEG phase separated above room temperature but below the reaction temperature. The final PEG percentage was 2% (w/v) for the reaction with the two PEG derivatives combined in a ratio of 1:1. Cu(II)SO₄ was added last to avoid premature crosslinking. Unless otherwise noted, the reaction was performed long enough such that the microspheres formed but aggregation was kept to a minimum. To halt the reaction, EDTA in PBS was added to the sample to chelate the copper. The microspheres were then centrifuged at 14,100 x g for 2 minutes and excess supernatant was removed. Na₂SO₄ and excess copper were removed by repeating this process two more times with 0.1 M EDTA and three times with PBS.

3.3.8 PEG₄-Azide/PEG₄-Alkyne Scaffold Formation

Scaffolds were formed from the washed PEG₄-azide/PEG₄-alkyne microspheres crosslinked with PEG-dithiol. Scaffold formation was performed by combining stock solutions of PEG-dithiol with the washed microspheres in a ratio of 1 vol PEG-dithiol per 1 vol of microspheres, and then 30% (w/v) dextran (M₉ ~100,000) in PBS was added to phase separate the PEG in the microspheres with 2 vol of dextran solution per 1 vol of microspheres/PEG-dithiol. The dextran/PEG microsphere solution was centrifuged at 1,000 x g for 10 minutes. The
thiol-yne photoaddition reaction was initiated with visible light from a Xenon arc lamp (Genzyme Focal Seal LS 1000; filtered at 480-520 nm) with 10 mM Eosin Y in PBS as the photoinitiator. The light was illuminated on the sample for at least 4 minutes.

3.3.9 RGD Peptide Attachment to PEG₄-Cyclooctyne

This was accomplished by reacting a cysteine-containing RGD sequence (Seq: GCYGRGDSPG; GenScript USA Inc.) with the cyclooctyne group on PEG with a thiol-yne photoaddition reaction. RGD was added to the PEG₄-cyclooctyne in a ratio of 1:8 RGD to cyclooctyne. A PEG solution was prepared with PEG₄-cyclooctyne, RGD, and 10 mM Eosin Y at 20% (w/v) in PBS. The process was initiated with visible light from a Xenon arc lamp with Eosin Y acting as the photoinitiator and was performed for at least 4 minutes. A stock solution of PEG₄-cyclooctyne with RGD was then used for microsphere and scaffold fabrication.

3.3.10 Michael-Type Addition of Thiols to Cyclooctyne Assay

PEG₄-cyclooctyne was tested to determine if a Michael-type addition might occur between the aza-dibenzocyclooctyne and thiols. Stock solutions of PEG₄-cyclooctyne and PEG-dithiol were combined in a ratio of two thiols per cyclooctyne. Solutions were left overnight on a 37°C heating block. Samples were checked the next day for bulk hydrogel formation.
3.3.11 PEG₄-Azide/PEG₄-Cyclooctyne Microsphere Fabrication

Stock solutions of PEG₄-azide and PEG₄-cyclooctyne (or PEG₄-cyclooctyne with RGD) were combined with PBS and Na₂SO₄. Unless otherwise noted, the concentration of Na₂SO₄ was chosen to lower the LCST of PEG such that the PEG phase separated above room temperature but below the reaction temperature. The final PEG percentage was 2% (w/v) for the reaction with the two PEG derivatives combined at a ratio of 1:1. One of the PEG derivatives was added last in the microsphere fabrication procedure to avoid premature crosslinking. Unless otherwise noted, the reaction was performed long enough such that the microspheres formed but aggregation was kept to a minimum. At the end of the reaction, three PBS washes as previously described for the PEG₄-azide/PEG₄-alkyne microsphere fabrication were performed to remove the Na₂SO₄. The microspheres were then used immediately or diluted by 15 times their volume with PBS to slow down their reaction kinetics and stored at 4°C.

3.3.12 PEG₄-Azide/PEG₄-Cyclooctyne Scaffold Formation

Scaffolds were formed from the washed PEG₄-azide/PEG₄-cyclooctyne microspheres. Scaffold formation was performed by adding a 30% (w/v) dextran solution to phase separate the microspheres with 2 vol of dextran solution per 1 vol of microspheres. The dextran/PEG microsphere solution was centrifuged at 1,000 x g for 10 minutes. No crosslinker was required because residual azide and cyclooctyne groups at the surface of the microspheres reacted with each other to crosslink the microspheres.
3.3.13 Cell Attachment and Spreading Assay

50 µL bulk gels were produced from a final concentration of 5% (w/v) PEG. The solution consisted of 6.25 µL PEG₄-azide stock solution, 6.25 µL PEG₄-cyclooctyne stock solution with or without RGD, and 37.5 µL of PBS and was used to coat most of the area of a 22 mm x 22 mm glass cover slip. The solutions were spread to the edges of the cover slip in order to wrap the hydrogel around the cover slip and prevent them from coming off. One set of cover slips had PEG with RGD, and another set of cover slips had PEG without RGD to serve as the control. The cover slips were coated with PEG and allowed to sit for 5 minutes. These glass cover slips were then placed in a 6-well plate, and 2 mL of endothelial growth media (EGM; MCDB 131 media supplemented with 10 µg/L epidermal growth factor, 10 mg/L heparin, 1.0 mg/L hydrocortisone, 0.2% antibiotic-antimycin, 2% fetal bovine serum, and 12 mg/L bovine brain extract). Each well was seeded with 1 x 10⁵ human aortic endothelial cells (HAEC) (Alford, Wang et al. 2010), and images were taken after 48 h using an Olympus IX70 microscope with an Olympus CPlanFl 10X/0.3 phase contrast objective.

3.3.14 Fabrication of PEG₄-Azide/PEG₄-Cyclooctyne Microsphere-Based Scaffolds with RGD in the Presence of Cells

Following the formation and washing of PEG₄-azide/PEG₄-cyclooctyne microspheres with RGD, the microspheres were centrifuged at 14,100 x g for 12 seconds to form a pellet and excess PBS was removed. This was done three times to remove all excess PBS. The microspheres were then resuspended in 200 µL of PBS with 500,000 HAEC. The resuspended
microspheres and the HAEC were formed into scaffolds with the addition of 30% (w/v) dextran solution as described above for the PEG₄-azide/PEG₄-cyclooctyne scaffold formation procedure. Once the scaffolds were made, excess dextran and PBS are replaced with 3 mL of EGM.

### 3.3.15 Fabrication of PEG₄-Azide/PEG₄-Cyclooctyne Bulk Hydrogels with RGD in the Presence of Cells

PEG₄-azide/PEG₄-cyclooctyne hydrogels with RGD were formed by combining 100 µL PEG₄-azide stock solution, 100 µL PEG₄-cyclooctyne with RGD stock solution with 500,000 HAEC resuspended in 200 µL of PBS. Once the hydrogels were made, they were washed with PBS and supplemented with 3 mL of EGM.

### 3.3.16 Cell Viability Assay for Scaffolds and Hydrogels

A cell viability assay was performed on the scaffolds and hydrogels that were formed in the presence of HAEC. A Live/Dead Viability/Cytotoxicity Kit (Invitrogen) was used to assess cell viability according to the manufacturer’s suggested protocol. Scanning confocal microscopy was performed on the scaffolds and hydrogels with 10X (0.45 DIC L WD 4.0) and 2X (0.1 WD 8.50) objectives using a Nikon Eclipse C1/80i scanning confocal microscope, and images were analyzed using EZ-C1 FreeViewer (Nikon Corporation). Images taken with the 10X objective were processed in MATLAB to determine percent cell viability.
3.3.17 Scaffold Analysis with Fluorescent Labeling

To image scaffolds that had been formed in the presence of cells, decellularization of the scaffolds was performed prior to labeling. Cells were lysed using deionized water. After inducing hypotonic shock for 30 minutes, the scaffolds were washed three times in PBS. Alexa Fluor 488-azide (Invitrogen) was used to label the remaining cyclooctyne on the microspheres that formed the PEG scaffolds. Labeling was done according to the manufacturer’s suggested protocol. Scanning confocal microscopy was performed on the scaffolds with 10X and 2X objectives as described earlier.

3.4 Results and Discussion

3.4.1 Formation of Click Microspheres

We investigated the formation of microspheres and porous scaffolds in the phase-separated state using copper(I)-catalyzed and copper-free azide-alkyne cycloadditions. For the copper(I)-catalyzed reaction, PEG₄-azide was mixed with PEG₄-alkyne, which was synthesized by the reaction of PEG₄-amine with propiolic acid. Reaction of PEG₄-azide with PEG₄-alkyne to form a bulk hydrogel at 37°C required the addition of Cu(II)SO₄ and sodium L-ascorbate (Figure 3.4a); however, some reaction was noted without copper at 95°C (results not shown). For the copper-free reaction at 37°C, PEG₄-azide was mixed with PEG₄-cyclooctyne (Figure 3.4b). The latter was synthesized by reaction of PEG₄-amine with a commercially available carboxyl-derivatized aza-dibenzocyclooctyne (Debets, van Berkel et al. 2010) (Figure 3.5). In both the
copper(I)-catalyzed and copper-free reactions, each four-arm PEG derivative served as a potential crosslink site (functionality = 4), forming highly crosslinked hydrogel networks upon reaction (Figure 3.4c). Reactive groups on the PEG monomers that are not consumed during microsphere formation should still be available for subsequent addition of biologically active molecules or further crosslinking (Scott, Nichols et al. 2010), which can be used for ‘bottom-up’ scaffold assembly (Figure 3.4c) (McGuigan and Sefton 2006; Fernandez and Khademhosseini 2010; Elbert 2011).
Figure 3.4. Clickable PEG microsphere formation. Clickable PEG derivatives were reacted using either: (a) copper(I)-catalyzed, or (b) strain-promoted Huisgen 1,3 dipolar cycloaddition between azides and alkynes. (c) In the presence of sodium sulfate, four-arm clickable PEG derivatives phase-separated and reacted to form highly crosslinked hydrogel microspheres. These microspheres contained residual reactive groups that allowed further crosslinking. (d) Microspheres were formed using copper(I)-catalyzed azide-alkyne cycloaddition. These microspheres were formed by inducing phase separation in 325 mM sodium sulfate upon heating to 37°C for 2 minutes. (e) Microspheres were formed using strain-promoted azide-aza-dibenzocyclooctyne cycloaddition. These microspheres were formed by inducing phase separation in 250 mM sodium sulfate upon heating to 37°C for 2 minutes. (f) and (g) Larger microspheres could be formed with the strain-promoted cycloaddition by inducing immediate phase separation at room temperature (25°C) with 500 mM sodium sulfate, mixing the solution by pipetting three times, and heating to 37°C for 2 minutes. Mixing in the phase-separated state resulted in the formation of much larger microspheres due to enhanced coalescence of PEG-rich domains prior to gelation.
Figure 3.5. The general mechanism behind the synthesis of clickable PEG derivatives. (a) The synthesis of PEG4-azide involved the nucleophilic azidation of PEG4-mesylate with sodium azide. (b) The synthesis of PEG4-alkyne involved the reaction of PEG4-amine and propiolic acid, following activation of the carboxylic acids by DIPCDI/HOBT. (c) The synthesis of PEG4-cyclooctyne was similar to the synthesis of PEG4-alkyne, but dibenzylcyclooctyne acid was substituted for propiolic acid.

To produce microspheres, the clickable PEG derivatives were reacted in the phase-separated state. Small microspheres (1-10 microns) were generated in the presence of sodium sulfate for both the copper(I)-catalyzed reaction (Figure 3.4d) and the copper-free reaction (Figure 3.4e). To form these small microspheres, the concentration of sodium sulfate was
chosen such that phase separation did not occur at room temperature, allowing mixing of the
reagents prior to the thermally induced phase separation. With the copper(I)-catalyzed reaction,
325 mM sodium sulfate resulted in phase separation upon heating from room temperature to
37°C. A 2 minute reaction was sufficient for microsphere formation. For the copper-free
reaction, a concentration of 250 mM sodium sulfate was needed for the formation of small
microspheres. The lower concentration of sodium sulfate was required because the LCST of
PEG was greatly depressed by the presence of the hydrophobic dibenzocyclooctyne on the PEG,
which was similar to an effect previously observed with acrylates and vinyl sulfones on PEG
(Nichols, Scott et al. 2009). Microspheres could also be formed by substituting the sodium
sulfate with dextran or polyacrylamide (mol wt 5 x 10⁶-6 x 10⁶; Polysciences, Inc.). Both of
these polymers form aqueous two-phase systems with PEG (Figure 3.6). With PEG₄-
cyclooctyne, much larger microspheres (e.g. diameters of 50 microns or greater) formed if higher
sodium sulfate concentrations were used. Higher sodium sulfate concentrations caused phase
separation of the PEG derivatives at room temperature, and the mixing of the reagents resulted in
the formation of larger droplets, presumably by flow-induced acceleration of coalescence (Figure
3.4f and 3.4g). The combination of large and small microspheres has been shown to produce
stronger materials than those formed from particles of uniform size (Van Tomme, van Nostrum
et al. 2008), and thus may be desirable and were further examined here.
Figure 3.6. Microsphere fabrication conditions. Microspheres could be formed using dextran or polyacrylamide instead of sodium sulfate to induce phase separation. PEG₄-azide/PEG₄-alkyne microspheres made using 0.12 mg copper with: (a) 10% dextran in PBS and formed at 37°C for 5 minutes, (b) 10% dextran in PBS and formed at 37°C for 10 minutes, (c) 2% polyacrylamide for 4 minutes at 70°C.
In contrast, the copper(I)-catalyzed reaction generally did not produce microspheres greater than 5 microns in diameter. A high concentration of sodium sulfate (650 mM Na$_2$SO$_4$) produced only small microspheres with the copper(I)-catalyzed system (Figure 3.7). Changes in the copper concentrations also did not affect microsphere size (Figure 3.8), nor did increases in the reaction temperature (Figure 3.9). This is somewhat surprising as faster reaction kinetics should result in smaller microspheres (Nichols, Scott et al. 2009). We have seen a similar result with photopolymerizations of PEG-diacrylate in which the presence of a precipitation polymerization was suggested to occur due to the enhanced solubility of the photoinitiator in the PEG-poor phase (Flake, Nguyen et al. 2011). It is possible that the copper ions also prefer the PEG-poor phase, such that a precipitation polymerization occurs in this phase, and very little crosslinking occurs in the PEG-rich droplets.
Figure 3.7. Varying reaction time for PEG4-azide/PEG4-alkyne microsphere fabrication. PEG4-azide/PEG4-alkyne microspheres made using 0.12 mg copper with 650 mM Na2SO4 in PBS mixed at 4°C and formed at 37°C. (a) Microspheres reacted for 2 minutes. (b) Microspheres reacted for 4 minutes. The reaction time of the PEG played an important role because if the reaction time was too short, no microspheres would be recovered, and if the reaction time was too long, the microspheres would begin to aggregate.
Figure 3.8. Varying copper concentration for PEG₄-azide/PEG₄-alkyne microsphere fabrication. PEG₄-azide/PEG₄-alkyne microspheres made using various concentrations of copper with 325 mM sodium sulfate in PBS and formed at 37°C for 2 minutes. (a) Microspheres fabricated with 0.03 mg of copper. (b) Microspheres fabricated with 0.06 mg copper. (c) Microspheres fabricated 0.12 mg of copper. Varying the copper concentration when forming microspheres in the presence of sodium sulfate appeared to have no effect on microsphere size. This suggested a precipitation polymerization was occurring.
Figure 3.9. Increasing reaction temperature for PEG₄-azide/PEG₄-alkyne microsphere fabrication. PEG₄-azide/PEG₄-alkyne microspheres made using 0.12 mg copper with 5% dextran in PBS and formed at 70°C. (a) Microspheres reacted for 3 minutes. (b) Microspheres reacted for 2.5 minutes.

3.4.2 Attachment of Cell Adhesion Peptides

We envision using microsphere-based scaffolds for the transplantation of vascular endothelial cells to achieve rapid vascularization following transplantation (Rouwkema, Rivron et al. 2008; Chen, Aledia et al. 2009). The high resistance of PEG materials to protein adsorption makes it necessary to functionalize the materials with cell adhesion peptides. A cysteine-containing RGD peptide (Seq: GCGYGRGDSPG) was used in a photoactivated thiol-yne reaction with PEG₄-cyclooctyne (Figure 3.10a). To determine the effectiveness of the RGD attachment protocol, a thin, non-porous bulk PEG hydrogel was formed from PEG₄-azide and PEG₄-cyclooctyne with about one RGD peptide per eight cyclooctyne groups. Endothelial cells seeded on bulk hydrogels without RGD peptide were unable to spread (Figure 3.10b). In contrast, endothelial cells seeded on hydrogels containing RGD peptide spread quite readily after 24 hours (Figure 3.10c). The success of attaching adhesion peptides indicates that other types of
peptides and molecules can be incorporated using photoactivated thiol-yne reaction for different functions, such as scaffold degradability.

![Diagram of thiol-yne reactions](image)

**Figure 3.10.** Attachment of cell adhesion peptide by thiol-yne reaction. (a) RGD peptide was added to PEG4-cyclooctyne via a thiol-yne photoaddition, reacting the thiol on a cysteine in the RGD peptide with aza-dibenzocyclooctyne on the PEG. To demonstrate attachment of functional RGD peptide, HAEC were seeded on PEG4-azide/PEG4-cyclooctyne bulk hydrogels. (b) Without RGD, the HAEC were not able to spread after 24 hours. (c) HAEC attached and spread on hydrogels containing RGD after 24 hours.

Michael-type addition of thiols to some activated alkynes is known (Waykole and Paquette 1989). To determine if a Michael-type addition might occur between the aza-dibenzocyclooctyne and thiols, PEG4-cyclooctyne was incubated with PEG-dithiol in solution at
a PEG concentration of 20% (w/v). This solution did not form a gel overnight at 37°C. However, a bulk gel formed within 4 minutes if Eosin Y was added to the solution and exposed to intense visible light (480-520 nm) (results not shown). This indicated that the thiol-yne reaction occurred readily, but that the Michael-type addition was insignificant.

### 3.4.3 Microsphere-Based Scaffold Formation

PEG microspheres were formed and then clicked together to form porous scaffolds using reactions that should not harm living cells. Due to the potential for copper toxicity (Baskin, Prescher et al. 2007), PEG₄-azide/PEG₄-alkyne microspheres were stitched together into a scaffold using a visible light-initiated thiol-yne reaction (Park, Kloxin et al. 2010) between PEG-dithiol and residual alkyne groups in the microspheres (Figure 3.11). This was further evidence of the specific nature of the thiol-yne reaction because microspheres formed with PEG₄-azide and PEG₄-alkyne and incubated with PEG-dithiol did not form scaffolds without photoinitiation (results not shown). Scaffolds with better handling properties were then formed by reacting PEG₄-azide/PEG₄-cyclooctyne microspheres directly with each other. We found that the crosslinking of the microspheres proceeded more efficiently in the phase-separated state (Smith, Segar et al. 2012), so a 500 µL mixture of microspheres and 500,000 HAEC was suspended on top of 500 µL of 30% (w/v) dextran (Figure 3.12a). PEG/dextran aqueous two-phase systems are widely used for cellular partitioning (Albertsson 1986), and toxicity was not expected to be a concern based on previous results with a cardiomyocyte cell line (Smith, Segar et al. 2012). Phase separation caused the microspheres to deswell and rapidly crosslink around the cells.
during centrifugation for 10 minutes at 1,000 x g. Following centrifugation, excess dextran and PBS were replaced with endothelial growth medium.

**Figure 3.11.** PEG₄-azide/PEG₄-alkyne microsphere-based scaffold formation. To form PEG₄-azide/PEG₄-alkyne microsphere-based scaffolds, microspheres were suspended 1:1 (v/v) in 20% PEG-dithiol. This mixture was then suspended 1:2 (v/v) in 30% dextran and centrifuged at 1,000 x g for 10 minutes. (a) Crosslinking of the microspheres was then performed with a thiol-yne photoaddition reaction. The process was initiated with visible light from a Xenon arc lamp (480-520 nm) with 10 mM Eosin Y in PBS as the photoinitiator. (b) Visible light was projected onto the microspheres at different angles for a total of 4 minutes to form a scaffold that was fragile but could be handled and manipulated with great care.
Figure 3.12. Formation of scaffolds in the presence of cells. (a) To form microsphere-based scaffolds, PEG microspheres were suspended 1:1 (v/v) in 30% dextran and centrifuged at 1,000 x g for 10 minutes. In 30% dextran, the PEG in the microspheres phase-separated from the dextran, causing the microspheres to deswell. This greatly enhanced reaction between the microspheres and resulted in stronger scaffolds. The scaffold formed a layer consisting of microspheres mixed with cells on top of the diluted dextran phase following centrifugation. Cells were incorporated into the scaffolds simply by combining them with the PEG microspheres in 30% dextran just prior to scaffold formation. (b) Once formed, PEG scaffolds supported their own weight and could be manipulated for routine cell culture. (c) A Live/Dead assay demonstrated 87.8% ± 3.5% viability of HAEC 2 days after scaffold formation. (d) Labeling of the microspheres with an azide-containing dye revealed a high degree of porosity within the scaffold.

Structural stability, high cell viability, and a network of interconnected pores are required for the eventual vascularization of the scaffold (Yang, Leong et al. 2001; Karageorgiou and Kaplan 2005). The formed scaffolds supported their own weight in solution and were able to be handled for cell culture and microscopy (Figure 3.12b). Two days after scaffold formation, the
viability of the HAEC within the scaffolds was calculated to be 87.8\% ± 3.5\% (Figure 3.12c). The smaller images shown to the right and bottom of Figure 3.12c are stacks of cross-sections in the y-z plane and x-z plane, respectively. These demonstrate that the cells are distributed in three dimensions within the scaffolds. A control was performed with PEG\textsubscript{4}-azide/PEG\textsubscript{4}-cyclooctyne bulk hydrogels with RGD made in the presence of cells. After 48 hours the calculated viability of the cells had dropped to 3.5\% ± 0.8\%. Confocal images taken of the hydrogel support this data (Figure 3.13). Comparatively, the microsphere-based scaffold performed much better than the standard bulk hydrogel. This is believed to be a result of a superior porous network structure in the microsphere-based scaffolds.
Figure 3.13. PEG₄-azide/PEG₄-cyclooctyne bulk hydrogel with RGD formed in the presence of cells 48 hours after fabrication. A Live/Dead assay demonstrated 3.5% ± 0.8% viability of HAEC 2 days after scaffold formation (live cells = green, dead cells = red).

To assess network pore structure, cells were removed from the scaffolds, and remaining cyclooctyne groups present in the scaffold were labeled with Alexa Fluor 488-azide dye. Figure 3.12d shows the high degree of porosity found in the scaffolds. Microspheres in some regions of Figure 3.12d do not appear to be connected due to the two dimensional nature of scanning confocal microscopy. The smaller images on the right and bottom of Figure 3.12d demonstrate
that the microspheres are in fact connected in three-dimensional space. Figure 3.12d also qualitatively demonstrates the abundant availability of unreacted groups on the microspheres following microsphere and scaffold formation. Unfortunately, due to the precise nature of fabricating microspheres, the density of the available unreacted groups on the microspheres cannot be readily adjusted without adversely affecting microsphere formation. If the reaction time is decreased, then the quantity of microspheres will be reduced or no microspheres will form (Figure 3.7a). If the reaction time between the PEG derivatives is extended, then the microspheres formed will undergo excessive aggregation, inhibiting scaffold formation later (Figure 3.7b). Following the removal of the cells, the scaffolds could still be handled and manipulated without breaking, further illustrating the connectivity of the microspheres.

3.5 Conclusions

We demonstrated in this study that highly porous scaffold materials can be produced by manipulating azide-alkyne cycloadditions and phase separations. The reactions were performed in two steps: one to produce hydrogel microspheres, and another to crosslink the microspheres with each other in the presence of cells. The high viability of endothelial cells during the scaffold formation process suggests that these materials are promising candidates for in vivo vascularization strategies. In contrast to bulk hydrogels, the cells are not technically encapsulated within the hydrogel, but rather are surrounded by a matrix of microspheres. We anticipate that such a strategy will yield more tissue-like architectures than bulk hydrogels due to the highly porous nature of the materials.
Chapter 4

Multifunctional Poly(ethylene glycol) Microspheres Used to Fabricate Modular Scaffolds for Vascularization

4.1 Abstract

The need for vascularization in tissue engineering has been an important challenge to overcome, since it is required to support cells in tissue-engineered constructs. One strategy to vascularize cells has been to use growth factor delivering scaffolds. The goal of this study was to produce scaffolds that promote vascularization via vascular endothelial growth factor (VEGF). Poly(ethylene glycol) (PEG) derivatives with multiple functional groups were used with two sequential aqueous two-phase systems to produce microsphere-based scaffolds for vascularization. Synthesized with multiple functional groups, these PEG derivatives were then formed into microspheres through phase separation and rapid gelation without the use of organic solvents or surfactants. These multifunctional microspheres, which contain clickable reactive groups, were then deswollen in dextran solutions and crosslinked together via their reactive click groups to form tightly packed porous scaffolds. The scaffolds were loaded with VEGF and

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1 Chapter 4 has been adapted from the following unpublished manuscript:
delivered it without loss of bioactivity. The scaffolds were also co-cultured with normal human lung fibroblasts (NHLFs) and endothelial colony-forming cell-derived endothelial cells (ECFC-ECs) to demonstrate their vascularization capabilities. After 21 days, the scaffolds demonstrated small regions of vascularization. The vascularization in these scaffolds was comparable to what we had observed with similar scaffold implants in mice. The multifunctional nature of the scaffolds enables integration of ideal features for vascularization, such as cell adhesion, controlled growth factor release, and adjustable mechanical properties. The versatility of these scaffolds may prove to be pivotal in forming extensive vascular networks for biomedical applications.

### 4.2 Introduction

The mechanisms of vascularization have been an important research endeavor, since numerous aspects of tissue engineering rely on supporting living cells and tissue. Tissue-engineered constructs that use or interact with living cells and tissues need to provide the means to carry out their biological functions. Cells and tissues further than 200 µm away from blood vessels have limited access to oxygen and nutrients and are unable to adequately remove carbon dioxide and cellular waste products (Carmeliet and Jain 2000; Rouwkema, Rivron et al. 2008; Novosel, Kleinhans et al. 2011; Auger, Gibot et al. 2013). Without a blood vessel network close enough to support cells and tissues, they are unable to function properly and eventually undergo cell death (Chu and Wang 2012; Auger, Gibot et al. 2013; Birbrair, Zhang et al. 2014). This problem can be solved through neovascularization, which primarily occurs through
vasculogenesis and angiogenesis. Vasculogenesis is the formation of new blood vessels without any preexisting vasculature, and angiogenesis is the formation of new blood vessels from preexisting vascularization (Auger, Gibot et al. 2013). Natural and synthetic biomaterials, such as poly(ethylene) glycol (PEG), poly(lactic-co-glycolic acid), fibrin gels, collagen, and Matrigel, have been used in tissue engineering approaches to form three-dimensional scaffolds to address this need for vascularization (Kim, Baez et al. 2000; Levenberg, Rouwkema et al. 2005; McGuigan and Sefton 2006; Chen, Aledia et al. 2009; Montaño, Schiestl et al. 2009; Chen, He et al. 2010; Du, Ghodousi et al. 2011; Elbert 2011). Synthetic biomaterials are especially promising because their properties can be easily modified for different tissue engineering applications.

PEG is an ideal synthetic biomaterial for an approach to vascularization of cells and tissues due to its many desirable properties. PEG is a hydrophilic polymer that has been shown to resist protein adsorption and non-specific biological interactions (Lee, Lee et al. 1995; Peppas, Keys et al. 1999; Alcantar, Aydil et al. 2000; Donahoe, Cohen et al. 2013) and can be modified to provide useful biological functions, such as cell adhesion and enzymatic biodegradation (West and Hubbell 1999; Lutolf and Hubbell 2005; Zhu 2010). PEG can also be modified to take advantage of a wide variety of chemical reactions including Michael-type addition and bioorthogonal click reactions (Huang, Michel et al. 2001; Agard, Prescher et al. 2004; Iha, Wooley et al. 2009). More recently, the versatility of PEG has expanded with the introduction PEG derivatives with more than a single type of functional group per PEG molecule (Obermeier, Wurm et al. 2011). While the properties of PEG have made it useful for bulk hydrogel and surface coating applications, one of the more strategic implementations of PEG for tissue engineering has been as modular or self-assembling hydrogel-based scaffolds with architecture
that better replicates the properties of natural tissues (Lutolf and Hubbell 2005; Scott, Nichols et al. 2010; Elbert 2011; Smith, Segar et al. 2012; Donahoe, Cohen et al. 2013; Nguyen, Snyder et al. 2013). Modular PEG scaffolds are comprised of different components that can be tailored to suit different types of applications.

Previously, modular PEG scaffolds were fabricated with PEG microspheres that were formed by phase inversion polymerization (Scott, Nichols et al. 2010; Elbert 2011; Smith, Segar et al. 2012; Nguyen, Snyder et al. 2013). The process was initiated by lowering the lower critical solution temperature (LCST) of reactive PEG derivatives with kosmotropic salts to induce thermal phase separation. They formed PEG-rich domains that coalesced and increased in size until they reached the gel point. The microspheres formed through this technique presented reactive groups on their surface that interacted with crosslinkers under favorable conditions to form modular PEG scaffolds. In this study, we synthesize PEG derivatives with more than one type of functional group (multifunctional PEG) to create PEG microspheres and scaffolds using the same techniques, but without the need for extra crosslinkers. The mechanical properties of these scaffolds were similar to those that were previously fabricated (Scott, Nichols et al. 2010; Smith, Segar et al. 2012), but with more adjustability. In addition, growth factors can be delivered from these scaffolds without affecting their bioactivity. We sought to demonstrate that these new types of scaffolds formed from multifunctional PEG could be used for vascularization applications.

4.3 Materials and Methods

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich.
4.3.1 PEG<sub>8</sub>-Vinyl Sulfone Synthesis

The synthesis procedure of eight-arm PEG-vinyl sulfone (PEG<sub>8</sub>-vinyl sulfone) from eight-arm PEG-OH (PEG<sub>8</sub>-OH; mol wt 10,000; Creative PEGWorks) was adapted from a procedure as previously described (Morpurgo, Veronese et al. 1996; Scott, Nichols et al. 2008). Solutions of PEG<sub>8</sub>-vinyl sulfone were prepared at 20% (w/v) in phosphate buffered saline (PBS; Pierce; pH 7.4) and sterile filtered with 0.22 µm syringe tip filters (Millipore).

4.3.2 PEG<sub>8</sub>-Amine Synthesis

The synthesis procedure of eight-arm PEG-amine (PEG<sub>8</sub>-amine) from eight-arm PEG-mesylate (PEG<sub>8</sub>-mesylate) was adapted from a procedure as previously described (Elbert and Hubbell 2001; Scott, Nichols et al. 2008). PEG<sub>8</sub>-mesylate was synthesized from PEG<sub>8</sub>-OH as described in the procedure for PEG<sub>8</sub>-vinyl sulfone synthesis (Morpurgo, Veronese et al. 1996; Scott, Nichols et al. 2008). Solutions of PEG<sub>8</sub>-amine were prepared at 20% (w/v) in PBS and sterile filtered with 0.22 µm syringe tip filters.

4.3.3 PEG<sub>8</sub>-Acrylate Synthesis

The synthesis procedure of eight-arm PEG-acrylate (PEG<sub>8</sub>-acrylate) from PEG<sub>8</sub>-OH was adapted from a procedure as previously described (Elbert and Hubbell 2001; Scott, Nichols et al.
Solutions of PEG₈-acrylate were prepared at 20% (w/v) in PBS and sterile filtered with 0.22 µm syringe tip filters.

4.3.4 PEG₈-Azide/Amine Synthesis

The synthesis procedure of eight-arm PEG-azide/amine (PEG₈-azide/amine) was adapted from a procedure for eight-arm PEG-azide (PEG₈-azide) synthesis as previously described (Nguyen, Snyder et al. 2013). PEG₈-azide was then partially reduced to PEG₈-amine by reduction of the azide groups to amine groups via Staudinger Reduction. The procedure was adapted from a previously described method (Wallace, Hanes et al. 2005). PEG₈-azide was dissolved in tetrahydrofuran (THF) and 1.15 equivalents of triphenylphosphine (Ph₃P) and 30 equivalents of ultrapure H₂O were added while on ice, and the reaction was allowed to go overnight under constant stirring and nitrogen flow. A large excess of H₂O to Ph₃P was needed for amine formation. Only enough Ph₃P and H₂O were added to reduce approximately 50% of the azide groups to amine groups. Excess THF and H₂O were removed by rotovapping, and PEG₈.azide/amine and triphenylphosphine oxide (TPPO) were precipitated out using cold diethyl ether. The product and byproduct were dried under vacuum overnight to remove any remaining diethyl ether. Once dry, the PEG₈-azide/amine and TPPO have cold toluene added, since TPPO is soluble in cold toluene and PEG is insoluble. The PEG₈-azide/amine was then vacuum filtered to remove the TPPO. The product then underwent the same extraction procedure with dichloromethane (DCM) that was done in the PEG₈-azide synthesis. ¹H NMR (300 MHz, CDCl₃, δ): 3.6 (PEG backbone), 2.9 (t, 2H, -CH₂CH₂NH₂). NMR of the product confirmed the reduction of some azides to amines with the amine feature at 2.9 ppm (Figure 4.1). Solutions of
PEG₈-azide/amine were prepared at 20% (w/v) in PBS and sterile filtered with 0.22 µm syringe tip filters.

**Figure 4.1.** NMR of PEG₈-azide/amine. PEG₈-azide/amine was synthesized using a three-step process. The PEG backbone is located at 3.6 ppm. The reduction of some azides to amines is shown by calculating the signal intensity for the amine peak at 2.9 ppm.

### 4.3.5 PEG₈-Cyclooctyne/Amine Synthesis

The synthesis procedure of eight-arm PEG-cyclooctyne/amine (PEG₈-cyclooctyne/amine) was adapted from a procedure for eight-arm PEG-cyclooctyne (PEG₈-cyclooctyne) synthesis as
previously described (Nguyen, Snyder et al. 2013). PEG₈-amine was synthesized as described previously. Then approximately half of the amines on PEG₈-amine were converted to cyclooctynes. PEG₈-amine was dissolved in DCM in a beaker, and 0.5 equivalents of diisopropylcarbodiimide (DIPCIDI) were added to a separate spherical flask with DCM while on ice and under nitrogen flow and constant stirring. Next, 0.5 equivalents of hydroxybenzotriazole (HOBt) and 0.5 equivalents of aza-dibenzo cyclooctyne with a pendant carboxylic acid (DBCO-acid; Click Chemistry Tools) were added to the mixture in the flask and allowed to stir for 10 minutes. While waiting, one equivalent of N,N-diisopropylethylamine (DIPEA) was added to the dissolved PEG₈-amine. Finally, this mixture was slowly added to the spherical flask, and the reaction was allowed to go for 24 hours in the ice bath, under constant stirring and nitrogen gas. Following that process, the urea precipitate was filtered out, and rotovapping, diethyl ether precipitation, and drying were performed. The product was then dissolved in distilled H₂O and underwent the same extraction procedure that was done for the PEG₈-amine. Further rotovapping, diethyl ether precipitation, and drying were done. ¹H NMR (300 MHz, CDCl₃, δ): 3.6 (PEG backbone), 5.4 (d, 2H, -CH₂-), 2.9 (t, 16H, -CH₂-NH₂). NMR of the product confirmed the conversion of some amines to cyclooctynes with the presence of a doublet at 5.4 ppm and the presence of amine groups with a triplet at 2.9 ppm (Figure 4.2). Solutions of PEG₈-cyclooctyne/amine were prepared at 20% (w/v) in PBS and sterile filtered with 0.22 µm syringe tip filters.
Figure 4.2. NMR of PEG₈-cyclooctyne/amine. PEG₈-cyclooctyne/amine was synthesized using a three-step process. The PEG backbone is located at 3.6 ppm. The partial conversion of amines to cyclooctynes is shown by the signal at 5.4 ppm. Some amines are still present shown by the signal at 2.9 ppm.

4.3.6 PEG₈-Azide/Amine/PEG₈-Vinyl Sulfone Microsphere Fabrication

The fabrication process of these structural microspheres was adapted from a procedure as previously described (Nguyen, Snyder et al. 2013). The PEG₈-azide/amine solution was combined with PEG₈-vinyl sulfone in a ratio of 1:1 azide/amine groups to vinyl sulfone groups.
The PEG₈-azide/amine/PEG₈-vinyl sulfone solution was diluted to 2% (w/v) at room temperature with PBS and 1.5 M sodium sulfate (Na₂SO₄) in PBS to reach a final concentration of 0.45M and volume of 500 µL. The concentration of Na₂SO₄ was chosen to lower the LCST of PEG such that the PEG phase separated above room temperature but below the reaction temperature. The solutions were then heated above the cloud point at 70°C for 5 minutes. To remove the Na₂SO₄, the microspheres were then buffer exchanged into PBS by diluting them 3:1 with PBS, titurating, and centrifuging at 14,100 x g for 2 minutes. This process was repeated two more times.

### 4.3.7 PEG₈-Cyclooctyne/Amine/PEG₈-Vinyl Sulfone Microsphere Fabrication

The fabrication process of these structural microspheres was adapted from a procedure as previously described (Nguyen, Snyder et al. 2013). The PEG₈-cyclooctyne/amine solution was combined with PEG₈-vinyl sulfone in a ratio of 1:1 cyclooctyne/amine groups to vinyl sulfone groups. The PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone solution was diluted to 2% (w/v) at room temperature with PBS and 1.5 M Na₂SO₄ in PBS to reach a final concentration of 0.45M and volume of 500 µL. The concentration of Na₂SO₄ was chosen to lower the LCST of PEG such that the PEG phase separated above room temperature but below the reaction temperature. The solutions were then heated above the cloud point at 70°C for 5 minutes. To remove the Na₂SO₄, the microspheres were then buffer exchanged into PBS by diluting them 3:1 with PBS, titurating, and centrifuging at 14,100 x g for 2 minutes. This process was repeated two more times.
4.3.8 Porogenic Microsphere Fabrication

The fabrication process was adapted from a procedure as previously described (Nguyen, Snyder et al. 2013). The PEG₈-cyclooctyne/amine solution was combined with different ratios PEG₈-vinyl sulfone and PEG₈-acrylate to tailor the porosity of microsphere scaffolds. Microspheres were created with a ratio of 2:1:1 cyclooctyne/amine to vinyl sulfone to acrylate groups unless otherwise stated. The PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone/PEG₈-acrylate solution was diluted to 2% (w/v) at room temperature with PBS and 1.5 M Na₂SO₄ in PBS to reach a final concentration of 0.45M and volume of 500 µL. The concentration of Na₂SO₄ was chosen to lower the LCST of PEG such that the PEG phase separated above room temperature but below the reaction temperature. The solutions were then heated above the cloud point at 70°C for 5 minutes. To remove the Na₂SO₄, the microspheres were then buffer exchanged into PBS by diluting them 3:1 with PBS, titurating, and centrifuging at 14,100 x g for 2 minutes. This process was repeated two more times.

4.3.9 Laminin Attachment to PEG Microspheres

The addition of cell adhesion peptide was done by mixing the PEG₈-azide/amine/PEG₈-vinyl sulfone microspheres and the PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone microspheres with laminin (Life Technologies) at 40 µg/mL and incubated at 4°C overnight. The cysteines on laminin react with available vinyl sulfone groups on the surface of the microspheres to covalently couple laminin to the microspheres. Microspheres are then buffered exchanged into
PBS three times by diluting them 3:1 with PBS, titurating, and centrifuging at 14,100 x g for 2 minutes.

4.3.10 Cysteine Capping of Excess Vinyl Sulfones

After laminin functionality was added to the microspheres, the microspheres were capped with cysteines to prevent any unreacted vinyl sulfone groups from inadvertently reacting later. The microspheres were incubated with L-cysteine at 2.5 mg/mL for 30 minutes at room temperature. Microspheres are then buffered exchanged into PBS three times by diluting them 3:1 with PBS, titurating, and centrifuging at 14,100 x g for 2 minutes.

4.3.11 PEG₈-Azide/Amine/PEG₈-Vinyl Sulfone/PEG₈-Cyclooctyne/Amine/PEG₈-Vinyl Sulfone Scaffold Formation

The scaffold fabrication process was adapted from a procedure as previously described (Smith, Segar et al. 2012; Nguyen, Snyder et al. 2013). Scaffolds were formed by combining PEG₈-azide/amine/PEG₈-vinyl sulfone microspheres and PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone microspheres in a ratio of 1:1 in the presence of 40% (w/v) dextran in PBS to phase separate the microspheres. Twice the volume of microspheres was added as dextran. The mixture was then centrifuged at 1,000 x g for 10 min.
4.3.12 PEG₈-Azide/Amine/PEG₈-Vinyl Sulfone/PEG₈-
Cyclooctyne/Amine/PEG₈-Vinyl Sulfone/PEG₈-Acrylate Porous Scaffold

Formation

The scaffold fabrication process was adapted from a procedure as previously described (Smith, Segar et al. 2012; Nguyen, Snyder et al. 2013). Scaffolds were formed by combining PEG₈-azide/amine/PEG₈-vinyl sulfone microspheres and PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone/PEG₈-acrylate microspheres in a ratio of 1:1 in the presence of 40% (w/v) dextran in PBS to phase separate the microspheres. Twice the volume of microspheres was added as dextran. The mixture was then centrifuged at 1,000 x g for 10 min. After two days hydrolysis of the ester bonds created a porous scaffold.

4.3.13 Scaffold Formation in the Presence of Fibroblast and Endothelial cells

Scaffolds formed in the presence of cells are fabricated in the same manner as described earlier under PEG₈-azide/amine/PEG₈-vinyl sulfone/PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone/PEG₈-acrylate scaffold formation. However, prior to the centrifugation step, cells are added to the microspheres. 2.5 million cells per mL of cord blood endothelial colony-forming cell-derived endothelial cells (ECFC-ECs; University of California, Irvine Medical Center) and 5 million cells per mL normal human lung fibroblasts (NHLFs; American Type Culture Collection) were added to the scaffolds for a total of 7.5 million cells per mL at a ratio of 2:1 NHLF to ECFC-EC. The mixture was then centrifuged at 200 x g for 10 min. Scaffolds were then placed in endothelial growth medium (EGM-2; Lonza) with 2% fetal bovine serum (FBS).
Scaffolds were maintained at 37°C in 100% humidified air containing 5% CO₂. The media was changed every other day for 21 days.

4.3.14 Fibrin-Based Tissue Constructs

Fibrin-based tissue constructs were fabricated using a variation of a previously described method (Chen, Aledia et al. 2009). Polydimethylsiloxane (PDMS; Dow Corning) chambers were prepared by mixing together PDMS and curing agent in a tissue culture dish (Corning) in a ratio of 10:1 and cured overnight in a vacuum chamber. Rings of PDMS with an inner diameter of 6 mm, an outer diameter of 8 mm, and a thickness of 1 mm were cut from the cured PDMS. The PDMS rings were then placed on 12 mm round glass cover slips (Ted Pella) to form the PDMS chambers. The PDMS chambers were sterilized with UV light for one hour. Next, fibrinogen was dissolved in Dulbecco’s phosphate-buffered saline (DPBS) at 10 mg/mL and sterile filtered with 0.22 µm syringe tip filters. NHLFs and ECFC-ECs were added in a ratio of 2:1 with the final cell concentration of 7.5 million cells per mL. 40 μL of the cell-fibrinogen mixture was added to each PDMS chamber and 2.4 μL of 50 U/mL thrombin was thoroughly mixed into the cell-fibrinogen mixture to form the fibrin-based tissue construct. The tissue constructs were then placed in EGM-2. Tissue constructs were maintained at 37°C in 100% humidified air containing 5% CO₂. The media was changed every other day for 21 days.
4.3.15 VEGF Bioactivity Assay

The VEGF bioactivity assay was adapted from a previously described endothelial cell migration assay (Alford, Kaneda et al. 2009; Alford, Wang et al. 2010). A monolayer of immortalized human aortic endothelial cells (HAEC-hT) (Alford, Wang et al. 2010) were cultured in 6-well tissue culture plates (Corning) with endothelial growth media (EGM; MCDB 131 media supplemented with 10 µg/L epidermal growth factor, 10 mg/L heparin, 1.0 mg/L hydrocortisone, 0.2% antibiotic-antimycin, 2% fetal bovine serum, and 12 mg/L bovine brain extract). Once the cells formed a monolayer, they were serum starved for 12 hours with low serum media (LSM; 0.1% fetal bovine serum and 1% antibiotic-antimycin). Scrape wounds were made using a 1 mL pipette tip to scrape the plates in the shape of a cross. Microspheres were loaded via diffusion with enough VEGF (Life Technologies) to deliver 10 ng/mL and then were crosslinked to form scaffolds. VEGF loaded scaffolds and scaffold controls were placed in the wells. Images were taken at day 0, 1, 2, and 3 using an Olympus IX70 microscope with an Olympus CPlanFl 10X/0.3 phase contrast objective. Wound gap was measured and processed using ImageJ software (NIH).

4.3.16 Heparin Attachment

Heparin attachment was adapted from a previously method (Roam, Nguyen et al. 2014). Heparin was incorporated into the scaffolds after the microsphere fabrication step. A solution of 500 mM N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC), 12.5 mM N-Hydroxy-succinimide (NHS), and 5% (w/v) heparin sodium salt in MES buffer (10 mM, pH 6.0)
was incubated at room temperature for 30 min. The activated heparin solution was added to the PEG₈-azide/amine/PEG₈-vinyl sulfone microspheres and PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone microspheres at an amine to heparin molar ratio of 20:1. The microspheres were incubated with the activated heparin solution for 30 min at room temperature. The microspheres were then buffer exchanged into PBS three times as previously described.

### 4.3.17 Mechanical Analysis of Scaffolds

Analysis of scaffolds using oscillatory shear rheometry (RFS3, Rheometric Scientific) was adapted as previously described (Smith, Segar et al. 2012). Standard scaffolds and porous scaffolds were fabricated as previously described. The scaffolds were approximately 10 mm in diameter and 2 mm thick. The scaffolds were characterized using 8 mm steel plates with the gap distance set to 1 mm to prevent slipping. A strain sweep established the extent of the scaffolds’ linearity to confirm that measurements were in the linear viscoelastic region. Frequency sweeps were then performed up and down the range of 0.1571 rads/s to 1.571 rads/s at 5% strain in order to obtain the storage modulus ($G'$).

### 4.3.18 Immunofluorescent Staining

Immunofluorescent staining was performed from a variation of a previously described process (Chen, Aledia et al. 2009; Chen, Aledia et al. 2010; White, Hingorani et al. 2012; White, Pittman et al. 2014). Scaffolds and fibrin-based tissue constructs were washed three times with
PBS and fixed in 10% formalin in PBS. The samples were then blocked in 2% (w/v) bovine serum albumin (BSA) in PBS at 4°C for an hour. The primary antibody, mouse anti-human CD31 (Abcam), was diluted 1:200 in 2% (w/v) BSA, and added to the samples for overnight incubation at 4°C. Samples were then washed in 2% (w/v) BSA for 20 minutes four times. The secondary antibody, Alexa Flour 488-labeled goat anti-mouse (Life Technologies), was diluted 1:500 in 2% (w/v) BSA, and added to the samples for overnight incubation at 4°C. Samples were then washed in 2% (w/v) BSA for 20 minutes four times. DAPI (Life Technologies) was added at 5 µg/mL and incubated for 30 min at room temperature. Finally, they were washed with 2% (w/v) BSA. Samples were stored in 10% formalin at 4°C when not in use.

4.3.19 Microscopy

Scanning confocal microscopy that was performed on the scaffolds and fibrin-based tissue constructs using either a Nikon Eclipse C1/80i scanning confocal microscope or an Olympus FV1200 laser scanning confocal microscope. Images from these microscopes were analyzed using EZ-C1 FreeViewer (Nikon Corporation) and FV10-ASW Viewer (Olympus Corporation), respectively. Brightfield microscopy was performed for the bioactivity assay using an Olympus IX70 microscope, and images were analyzed with ImageJ.
4.3.20 Statistical Analysis

The results of quantification are reported as a mean ± standard deviation. Statistical comparisons between conditions were performed using one-way ANOVA with a post hoc Tukey test. Results were considered statistically significant if \( p < 0.05 \).

4.4 Results and Discussion

4.4.1 Multifunctional PEG Synthesis

We developed a method for synthesis of eight-arm PEG with two types of functional groups on a single PEG molecule. This development of the synthesis of PEG₈-azide/amine and PEG₈-cyclooctyne/amine builds upon previous work from our laboratory (Nguyen, Snyder et al. 2013). Synthesis of PEG₈-azide/amine required only one additional step after the PEG₈-azide was produced. PEG₈-azide/amine was made by partially reducing the azide groups to amine groups via Staudinger reduction (Wallace, Hanes et al. 2005). In order to obtain approximately an equal ratio of azide and amine groups, 1.15 equivalents of Ph₃P and 30 equivalents of ultrapure H₂O were needed for the reaction. Several attempts were required to optimize the procedure and reach the final ratio, since the equivalents used in established methods are normally designed to react all available azide groups. Calculation of the NMR peak of the amines at 2.9 ppm with regard to the PEG backbone at 3.6 ppm verified that the final product that was approximately 50% azide and 50% amine (Figure 4.1). Synthesis of PEG₈-cyclooctyne/amine was simpler in that only required a modification of the step converting
amines into cyclooctynes. In order to obtain approximately an equal ratio of cyclooctyne and amine groups, only 0.5 equivalents of DIPCDI, HOBt, DIPEA, and DBCO-acid were needed for the reaction. The procedure went through several rounds of optimization in order to obtain a product that had approximately an equal number of cyclooctyne and amine groups. Calculation of the NMR peak of the amines at 2.9 ppm and the cyclooctynes at 5.4 ppm with regard to the PEG backbone at 3.6 ppm confirmed the final product had approximately and equal ratio of cyclooctynes to amines (Figure 4.2).

4.4.2 Microsphere and Scaffold Fabrication

Microsphere and scaffold fabrication was adapted from methods that had been previously developed (Scott, Nichols et al. 2008; Scott, Nichols et al. 2010; Nguyen, Snyder et al. 2013) and combined them in a new way to improve upon modular PEG scaffolds. Previously, microspheres were fabricated by lowering the LCST of PEG with Na₂SO₄ to thermally induce phase separation and create spherical PEG-rich domains that coalesced until they reached the gel point and form microspheres (Scott, Nichols et al. 2008; Nichols, Scott et al. 2009; Scott, Nichols et al. 2010; Nguyen, Snyder et al. 2013). This method was still used to create microspheres by combining PEG₈-azide/amine with PEG₈-vinyl sulfone or PEG₈-cyclooctyne/amine with PEG₈-vinyl sulfone in 450 mM Na₂SO₄ at 70°C for 5 minutes. These combinations created the structural microspheres PEG₈-azide/amine/PEG₈-vinyl sulfone and PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone (Figure 4.3). Unreacted vinyl sulfones on the surface of the microspheres can be used to add desired functionalities to the microspheres. The microspheres were capped with L-cysteine once all desired functionalities were added. The
reactive click groups on the microspheres were used to form scaffolds from the microspheres via PEG/dextran aqueous two-phase system as described previously (Elbert 2011; Smith, Segar et al. 2012; Nguyen, Snyder et al. 2013). In dextran, microspheres deswelled and rapidly crosslinked during centrifugation at 1,000 x g for 10 minutes (Figure 4.4).

**Figure 4.3.** Fabrication of structural PEG microspheres. PEG₈-cyclooctyne/amine or PEG₈-azide/amine was combined with PEG₈-vinyl sulfone in 450 mM Na₂SO₄ at 70°C for 5 minutes. Unreacted vinyl sulfones on the surface of the microspheres can be used to add desired functionalities to the microspheres prior to being capped with L-cysteine.
Figure 4.4. Fabrication of PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone/PEG₈-azide/amine/PEG₈-vinyl sulfone scaffolds. (a) Macroscopic image of PEG scaffold. (b) PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone microspheres were mixed and centrifuged with PEG₈-azide/amine/PEG₈-vinyl sulfone microspheres in 40% (w/v) dextran at 1,000 x g for 10 minutes. Phase separation caused microspheres to deswell and the click reactive groups crosslinked together to form stable scaffolds.
Porogenic microspheres could be created in the same fashion by combining PEG₈-azide/amine or PEG₈-cyclooctyne/amine with PEG₈-acrylate instead of PEG₈-vinyl sulfone in 450 mM Na₂SO₄ at 70°C for 5 minutes. These combinations created the porogenic microspheres PEG₈-azide/amine/PEG₈-acrylate and PEG₈-cyclooctyne/amine/PEG₈-acrylate. However, for the sake of simplicity we only created and used PEG₈-cyclooctyne/amine/PEG₈-acrylate porogenic microspheres (Figure 4.5). The amount of porosity introduced into the scaffold can be tailored by the addition of PEG₈-vinyl sulfone along with PEG₈-acrylate. The ratio of PEG₈-vinyl sulfone to PEG₈-acrylate controls the porosity. A higher ratio of PEG₈-vinyl sulfone adds more structural stiffness while more PEG₈-acrylate adds more porosity. Combining PEG₈-cyclooctyne/amine, PEG₈-acrylate, and PEG₈-vinyl sulfone produced mainly PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone/PEG₈-acrylate microspheres with the possible formation of PEG₈-cyclooctyne/amine/PEG₈-acrylate microspheres and PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone microspheres (Figure 4.6). Vinyl sulfones were capped with L-cysteine after all desired functionalities were added. The reactive click groups on the microspheres were used to form porous scaffolds from the combination of these microspheres with the structural microspheres via PEG/dextran aqueous two-phase system. Microspheres deswelled in 40% (w/v) dextran and rapidly crosslinked during centrifugation at 1,000 x g for 10 minutes (Figure 4.7). Within 48 hours the porogenic microspheres dissolved leaving a porous scaffold. These porous scaffolds are similar to ones previously created (Scott, Nichols et al. 2010), but they did not require the addition of serum proteins for crosslinking into scaffolds because of the added click functionality. As a result, these scaffolds have increased biocompatibility to make them more suitable as implants for vascularization.
**Figure 4.5.** Fabrication of porogenic PEG microspheres. PEG₈-cyclooctyne/amine was combined with PEG₈-acrylate in 450 mM Na₂SO₄ at 70°C for 5 minutes. These microspheres dissolved within two days if left in an aqueous environment.
Figure 4.6. Tailoring of porogenic PEG microspheres for porous scaffold fabrication. PEG₈-cyclooctyne/amine was combined with PEG₈-acrylate and PEG₈-vinyl sulfone at 450 mM Na₂SO₄ at 70°C for 5 minutes. Adjusting the ratio of acrylates to vinyl sulfones increased or decreased the amount of porosity that was introduced into the scaffolds. This process produced mainly microspheres with both acrylates and vinyl sulfones (PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone/PEG₈-acrylate microspheres) with the possible formation of structural microspheres (PEG₈-cyclooctyne/amine/PEG₈-acrylate microspheres) and porogenic microspheres (PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone microspheres).
**Figure 4.7.** Fabrication porous scaffolds. (a) Macroscopic image of porous PEG scaffold. (b) PEG₈-cyclooctyne/amine/PEG₈-acrylate microspheres were mixed and centrifuged with PEG₈-azide/amine/PEG₈-vinyl sulfone microspheres in 40% (w/v) dextran at 1,000 x g for 10 minutes. Phase separation caused microspheres to deswell, and the click reactive groups crosslinked together to form stable scaffolds. Within 48 hours the porogenic microspheres dissolved leaving a porous scaffold. Using porogenic microspheres found in Figure 4.6 yielded similar results.

### 4.4.3 Scaffold Mechanical Properties

Scaffolds created by previous work (Scott, Nichols et al. 2008; Scott, Nichols et al. 2010) had storage moduli that were an order of magnitude lower than those of bulk PEG hydrogels,
making them delicate. However, they held together if carefully handled and had storage moduli that were lower than heart tissue, similar to liver tissue, and higher than collagen gels or Matrigel (Raeber, Lutolf et al. 2005; Cullen, Lessing et al. 2007; Levental, Georges et al. 2007; Lai, Li et al. 2008; Jiao, Clifton et al. 2012). The stiffness of these scaffolds was limited by their fabrication process until PEG/dextran aqueous two-phase system was implemented to fabricate the modular scaffolds (Elbert 2011; Smith, Segar et al. 2012; Nguyen, Snyder et al. 2013). The scaffolds fabricated using this system could be made to match the storage moduli of heart tissue (Jiao, Clifton et al. 2012; Smith, Segar et al. 2012). Scaffolds made from multifunctional PEG derivatives demonstrated a range of storage moduli. Scaffolds consisting of only structural microspheres exhibited a storage moduli greater than scaffolds previously made ($G' = 700 – 1,000$ Pa) using the PEG/dextran aqueous two-phase system and closer to that of PEG hydrogels (Figure 4.8) (Scott, Nichols et al. 2010; Roberts, Earnshaw et al. 2011; Smith, Segar et al. 2012). Porous scaffolds formed with an equal amount of structural and porogenic microspheres had a storage modulus that was comparable to modular scaffolds created by crosslinking microspheres with serum proteins ($G' = 200 – 400$ Pa) (Figure 4.9) (Scott, Nichols et al. 2010). The rheometry data suggests that scaffolds can be tailored to varying degrees of stiffness within the range of $G'$ of approximately 400 to 1,400 Pa by adjusting the amount of porogenic microspheres incorporated into the scaffolds.
Figure 4.8. Rheometry data of nonporous PEG microsphere scaffold. PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone/PEG₈-azide/amine/PEG₈-vinyl sulfone scaffolds had a storage moduli ($G'$) greater than scaffolds previously made with serum proteins or the PEG/dextran aqueous two-phase system.
Figure 4.9. Rheometry data of porous PEG microsphere scaffold. PEG₈-cyclooctyne/amine/PEG₈-acrylate/PEG₈-azide/amine/PEG₈-vinyl sulfone scaffolds had a storage moduli ($G'$) comparable to scaffolds previously made with serum proteins.

4.4.4 VEGF Delivery and Bioactivity

In order to achieve better vascularization in the modular PEG scaffolds, VEGF was loaded into the multifunctional PEG microspheres before being crosslinked into scaffolds and their bioactivity was tested after being released from the scaffolds. PEG₈-azide/amine/PEG₈-vinyl sulfone and PEG₈-cyclooctyne/amine/PEG₈-vinyl sulfone microspheres were loaded with VEGF and centrifuged in the presence of dextran to form scaffolds. Enough VEGF was loaded to deliver 10 ng/mL of VEGF for the bioactivity assays. HAEC-hTs were cultured in 6-well plates until they formed a monolayer. The cells were serum starved in LSM for 12 hours before the scrape wound assay was performed. Scaffolds delivering VEGF and scaffold controls were
placed in the wells with scrape wounds, and scrape wound distances were measured for four
days. Scaffolds that did not deliver VEGF did not display a significant change in wound width
(ANOVA and a Tukey post-hoc test; Figure 4.10). Scaffolds that were loaded with VEGF
showed a significant change ($p < 0.05$) in wound width in next three days when compared to Day
0 (Figure 4.11). This suggests that VEGF remained active after being loaded and released from
the scaffolds. Thus, the bioactivity of VEGF was not affected by the microsphere and scaffold
fabrication process.

Figure 4.10. Wound width of scaffolds without VEGF delivery. The scrape wound assay
revealed no significant changes in wound width from scaffolds without VEGF after three days.
Figure 4.11. Wound width of scaffolds delivering VEGF. The scrape wound assay revealed a significant difference ($p < 0.05$) in wound width when comparing days 1, 2, and 3 to day 0. This indicated that the bioactivity of VEGF from these scaffolds was still present after being loaded and released.

We aimed to control the release of VEGF from the scaffolds by incorporating heparin into the microspheres. We chose heparin to control the release of VEGF, since it has been shown to specifically bind a variety of proteins, such as growth factors, cytokines, and enzymes (Sasisekharan and Venkataraman 2000; Capila and Linhardt 2002; Knaack, Lode et al. 2014). More specifically, it has been shown that heparin controlled release of VEGF improved angiogenesis and blood vessel maturation (Steffens, Yao et al. 2004; Nillesen, Geutjes et al. 2007; Chen, He et al. 2010; Knaack, Lode et al. 2014). In order to incorporate heparin into the scaffolds, heparin was activated with EDC/NHS and reacted with the amines on the PEG$_8$-azide/amine/PEG$_8$-vinyl sulfone and PEG$_8$-cyclooctyne/amine/PEG$_8$-vinyl sulfone microspheres (Figure 4.12). The microspheres were loaded with VEGF as was described and crosslinked.
together in dextran to form scaffolds. The scrape wound assay was performed on these scaffolds to determine if heparin had an effect on VEGF delivery in these scaffolds. The results from the assay depict a decreasing wound width trend, but the differences in wound widths were not found to be statistically significant ($p > 0.05$) (Figure 4.13). Since previous results demonstrated that VEGF bioactivity was not affected by the scaffolds fabrication process and other studies have demonstrated functional controlled release of VEGF with heparin, we concluded that not enough VEGF was released to make a statistically significant difference in wound width. Heparin may have decreased the burst release of VEGF from the scaffolds, reducing the amount of delivered VEGF needed to promote endothelial cell migration. The results indicate that the controlled release of VEGF in the heparinated scaffolds needs to be modified and measured in order to achieve a controlled burst release of VEGF that is needed to promote vascularization.
Figure 4.12. Heparin attachment onto PEG microspheres. Heparin was activated with EDC/NHS and added to the microspheres to react with the available amine groups. The heparinated microspheres were then loaded with VEGF and centrifuged in the presence of dextran to form scaffolds.
Figure 4.13. Wound width of heprinated scaffolds delivering VEGF. The scrape wound assay revealed a trend in decreasing wound width when comparing days 1, 2, and 3 to day 0. However, the differences in wound width were not statistically significant ($p > 0.05$).

4.4.5 Vascularization of PEG Scaffolds

Porous scaffolds were fabricated in the presence of cells to assess their ability to support vascularization. The PEG/dextran aqueous two-phase system described earlier was used to form scaffolds in the presence of cells because they have been widely used for cellular partitioning and do not adversely affect cells (Albertsson 1986; Smith, Segar et al. 2012; Nguyen, Snyder et al. 2013). NHLFs and ECFC-ECs were used to vascularize the scaffolds because they have been successfully used in fibrin-based tissue constructs and fibrin gels to create blood vessel networks (Chen, Aledia et al. 2009; Moya, Alonzo et al. 2014). PEG$_8$-azide/amine/PEG$_8$-vinyl sulfone microspheres were chosen to provide the structural support needed for the scaffold. To add porosity to the scaffold, porogenic PEG$_8$-cyclooctyne/amine/PEG$_8$-vinyl sulfone/PEG$_8$-acrylate
microspheres were fabricated with a ratio of 1:1 vinyl sulfone to acrylate. This ratio was chosen to make the scaffold porous but not too delicate to handle. Cell adhesion peptide was added by mixing the microspheres with laminin and incubating overnight. Laminins are proteins in the extracellular matrix, and they make up part of the basement membrane. Laminin contains the peptide sequence RGD in its alpha-chain that promotes adhesion of endothelial and fibroblast cells (Couchman, Höök et al. 1983; Beck, Hunter et al. 1990). Following laminin attachment and capping of excess vinyl sulfone groups, the microspheres were buffer exchanged, and NHLFs and ECFC-ECs were added to the microspheres. The mixture of cells and microspheres was combined with dextran and centrifuged to form cell-based PEG scaffolds. Within two days, the porogenic microspheres dissolved to increase scaffold porosity. Scaffolds were cultured for 21 days before they were analyzed. Fibrin-based tissue-engineered constructs were also made concurrently with the scaffolds to serve as a positive control. Fibrin gels with NHLFs and ECFC-ECs were formed in PDMS chambers to create the fibrin-based tissue-engineered constructs. They were cultured for 21 days, after which both the scaffolds and fibrin-based tissue-engineered constructs were fixed and immunofluorescently stained to assess vascularization with confocal microscopy. Both the fibrin-based tissue constructs and the PEG scaffolds had vascularization after 21 days (Figure 4.14). The fibrin-based tissue constructs had extensive vascularization while the porous scaffolds were limited to small regions of vascularization. The vascularization of the fibrin-based tissue constructs was expected, since NHLFs and ECFC-ECs have been shown to migrate through fibrin gels and form vascular networks (Brown, Lanir et al. 1993; Lafleur, Handsley et al. 2002; Chen, Aledia et al. 2009; Moya, Alonzo et al. 2014). Since the porous PEG scaffolds can be extensively modified, the vascularization results proved to be very promising for the potential of these scaffolds for
vascularization. Making adjustments to the porosity of the scaffolds and incorporating biodegradability into these scaffolds are just a few of the modifications that can be made to improve vascularization within the scaffold. We anticipate that several strategies can be used to improve the PEG scaffolds to have them match the vascularization found in fibrin-based tissue constructs.
**Figure 4.14.** Immunofluorescent images of fibrin-based tissue constructs and porous PEG scaffolds with lamin after co-culturing NHLFs and ECFC-ECs for 21 days (ECFC-ECs = green, nuclei = blue). (a) Vascularization was prominent in the fibrin gels after 21 days. (b) Vessel formation was found in the porous PEG scaffolds in a few small regions after 21 days.
4.5 Conclusions

In this study we demonstrated the fabrication of microspheres from multifunctional PEG derivatives and crosslinked them together to form porous modular PEG scaffolds for vascularization. The multifunctional nature of the PEG derivatives allowed for simple modification with additional functionalities, such as cell adhesion and VEGF delivery, while maintaining biocompatibility. The scaffolds were fabricated to exhibit a range of mechanical stiffness by changing the composition of the porogenic and structural microspheres. They could also be loaded with VEGF and deliver the growth factor without losing bioactivity. Finally, coculturing fibroblasts and endothelial cells in the porous PEG scaffolds demonstrated that they could provide a potential environment for vascularization. We anticipate that the addition of more functionality, such as degradability, to the porous PEG scaffolds can improve their ability to support vascularization.
Chapter 5

Conclusions

5.1 Summary of Dissertation

An ongoing challenge in tissue engineering has been to develop ways of supporting cells and tissues in tissue-engineered constructs and scaffolds. Vascularization has been studied as a solution to this problem by directing blood flow to cells and tissues. The goal of this work was to fabricate porous scaffolds that delivered growth factors and directed angiogenesis into the scaffolds. In Chapter 2, we created porous modular PEG microsphere-based scaffolds for *in vivo* vascularization. Reactive PEG derivatives were synthesized, and microspheres were made by exploiting the LCST of PEG. Microspheres were made to create pores, provide structural support, provide cell adhesion, and deliver S1P. These microspheres were crosslinked together with BSA to form scaffolds and were implanted in mice. We removed them after 14 days to assess how well the scaffolds induced angiogenesis. Photoacoustic microscopy revealed that scaffolds delivering S1P demonstrated greater recruitment of blood vessels than control scaffolds. This was confirmed with H&E staining of scaffold sections. However, the H&E staining also revealed that the scaffolds caused foreign body response. This was mainly attributed to the BSA used to control the delivery of S1P and crosslink the microspheres into scaffolds. Since BSA was needed to fabricate the scaffolds, we devised a new way to crosslink PEG microspheres into scaffolds.
In Chapter 3, we synthesized new PEG derivatives with click functionalities because of their bioorthogonal properties. Click reactions are highly specific reactions that do not have unwanted interactions with other proteins and biomolecules. We also replaced BSA with a PEG/dextran aqueous two-phase system to crosslink the microspheres into scaffolds. Microspheres and scaffolds were initially made with copper catalyzed click reactions and thiol-yne photopolymerizations. However, the copper catalyst was toxic to cells, so we used strain-promoted click reactions. This change removed any potential toxicity and simplified the fabrication of the microspheres and scaffolds, since we could now make them without a copper catalyst and photopolymerizable crosslinker molecule. RGD peptide was incorporated into the scaffolds for cell adhesion through thiol-yne photopolymerization. We were able to form scaffolds in the presence of endothelial cells without affecting the viability of the cells.

In Chapter 4, we modified the PEG derivatives synthesized in Chapter 3 to make them multifunctional PEG derivatives. These PEG derivatives were synthesized to create more options for fabricating and functionalizing microspheres and scaffolds. We fabricated microspheres, crosslinked them into scaffolds, and loaded them with VEGF. They were shown to deliver VEGF without affecting its bioactivity. Scaffolds were also modified with heparin to control the release of VEGF, but their ability to deliver VEGF effectively was inconclusive. The scaffolds were fabricated in the presence of fibroblasts and endothelial cells to test their ability to support vascularization. The scaffolds supported small regions of vascularization, but it was not as prominent as the vascular network found in fibrin gel controls. With some modifications, these scaffolds hold a great deal of promise for vascularization in tissue engineering.
5.2 Future Directions

The modular microsphere-based PEG scaffolds presented in Chapter 4 are promising candidates for promoting vascularization. Several improvements can be made to increase their vascularization potential. While we demonstrated that the scaffolds did not affect the bioactivity of VEGF, the results from VEGF delivery from heparinated scaffolds were ambiguous. One possibility was that there was not enough VEGF loaded into the heparinated microspheres because the heparin attached to the surface of the microspheres prevented adequate loading of the VEGF into the microspheres for release. The heparinated microspheres could also have been adequately loaded with VEGF, but the amount of heparin attached on the microspheres prevented an adequate burst release of VEGF as found with scaffolds delivering VEGF without heparin. A solution to this is to measure and compare VEGF release from scaffolds with and without heparin using an enzyme-linked immunosorbent assay (ELISA) and adjusting the amount of heparin attached to heparinated scaffolds to improve VEGF loading and delivery for endothelial cell migration. Different VEGF loading times can be performed on heparinated microspheres, and VEGF release can be measured from scaffolds formed by those microspheres to determine what effect loading times have on burst and total VEGF release. An ELISA can also be used to measure VEGF release from scaffolds with varying degrees of heparin incorporation to compare their burst release of VEGF to scaffolds without heparin. These strategies will help promote vascularization by improving VEGF release from heparinated scaffolds.

In an effort to decrease the foreign body response we observed in Chapter 2, we removed BSA from the scaffolds loaded with S1P as presented in Chapter 4. However, this had a
negative effect on the delivery of S1P. Since S1P has been shown to play an important role in vascularization, it is important to incorporate their delivery back into the scaffolds. An alternative method for delivering S1P may be the use PLGA. PLGA microparticles have been used for sustained delivery of S1P to promote angiogenesis and vascular maturation in mice (Qi, Okamoto et al. 2010). Copolymers of PLGA-PEG have been synthesized as a drug delivery system by activating PLGA with EDC/NHS and conjugating it with the amine on COOH-PEG-NH₂ (Kumara, Sahoo et al. 2014; Kumar, Sahoo et al. 2015). This technique can be modified to create PLGA-PEG microspheres that encapsulate S1P and crosslink via click reactions with PEG microspheres to form scaffolds. The degradability of the PLGA-PEG microspheres could also control the release of S1P and increase the porosity of the scaffold over time, removing the need for additional porogenic microspheres. Achieving delivery of S1P and VEGF simultaneously from the scaffold may increase the success of scaffold vascularization in vivo.

While the vascularization of scaffolds presented here is promising, more development must be done to produce optimal scaffolds. One solution would be to increase scaffold porosity; however, this would be at the cost of scaffold stiffness. A more promising approach is to incorporate degradable peptides and proteins into the scaffold to allow cell degradability. Matrix metalloproteinases (MMPs) have been shown to be produced by endothelial cells to degrade basement membranes for proliferation and migration during the process of angiogenesis (Zucker, Conner et al. 1995; Sang 1998; Lafleur, Handsley et al. 2002). MMPs are a family of zinc-dependent endopeptidases that degrade extracellular matrix (ECM) proteins, such as laminin, collagen, and fibronectin (Sang and Douglas 1996; Sang 1998). Introduction of MMP degradable peptide and proteins into the structure of the scaffold would allow endothelial cells to break down scaffold to suit their needs. This can also be achieved by making laminin an integral
part of the structure of the scaffolds. For example, laminin can be conjugated to PEG to create PEG-laminin microspheres that can also crosslink with structural PEG microspheres in the presence of cells to form scaffolds. Like the MMP degradable peptide, this will allow endothelial cells to degrade the scaffold to suit their needs.

Once these issues have been addressed and tested in vitro, the PEG microsphere scaffolds can be tested in vivo as was done in Chapter 2. After these scaffolds successfully promote vascularization in vivo, the next step will be to apply them in scenarios that require development of a vascular network for cell and tissue survival. Successful vascularization techniques will someday lead to better applications of tissue-engineered constructs and tissue implants. The goal is that these applications will become reliable ways to replace or repair damaged and defective tissues.
References


and tissue-specific considerations." European Spine Journal 17(Suppl 4): 467-479.

Chen, L., Z. He, et al. (2010). "Loading of VEGF to the heparin cross-linked demineralized bone
matrix improves vascularization of the scaffold." Journal of Materials Science: Materials


Accelerates the Formation of Functional Anastomosis with Host Vasculature." Tissue

Derived Vessels with Host Vasculature Is Promoted by a High Density of Cotransplanted


Clark, E. R. and E. L. Clark (1939). "Microscopic observations on the growth of blood
capillaries in the living mammal." American Journal of Anatomy 64(2): 251-301.

Clark, M. and P. Kiser (2009). "In situ crosslinked hydrogels formed using Cu(I)-free Huisgen
cycloaddition reaction." Polymer International 58(10): 1190-1195.

Cardiovascular Research 49(3): 507-521.


Crescenzi, V., L. Cornelio, et al. (2007). "Novel hydrogels via click chemistry: synthesis and
potential biomedical applications." Biomacromolecules 8(6): 1844-1850.

Cullen, D. K., M. C. Lessing, et al. (2007). "Collagen-Dependent Neurite Outgrowth and
Response to Dynamic Deformation in Three-Dimensional Neuronal Cultures." Annals of

denzyme PEGylation via copper-free (3+2) cycloaddition." Chemical Communications


English, D., Z. Welch, et al. (2000). "Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and
provides a novel link between hemostasis and angiogenesis." The FASEB Journal 14(14): 2255-2265.


