EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF ELASTIN-LIKE POLYPEPTIDES CONTAINING CHONDROITIN SULPHATE BINDING DOMAINS

by

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Abstract

The development of small-diameter artificial blood vessels that mimic the properties of natural blood vessels has proven to be a clinical challenge. While autologous vessels are the standard, they can be difficult to obtain and require invasive surgeries. Synthetic materials have been successful in large diameter applications, but they have been unsuccessful in small-caliber environments due to a number of factors including thrombus formation, intimal hyperplasia, and infection. Intimal hyperplasia, of particular interest in this study, involves the build up of smooth muscle cells (SMCs) in the intimal layer of the artery due to abnormal migration and proliferation.

This work focuses on the development of a new polymer that has the potential to function as an intimal/medial component of a small-diameter blood vessel. Using recombinant elastin-like polypeptides (ELPs) developed by the Woodhouse laboratory, as well as chondroitin sulphate-specific binding sequences (CSBD1 and CSBD2) determined by the Panitch laboratory, a new elastin-like polypeptide-chondroitin sulphate binding domain (ELP-CSBD) block copolymer has been developed and characterized. The expression of the ELP1-CSBDs was accomplished using *E. coli* BL21 cells in a bioreactor or shaker flask systems. The polypeptides were purified using dialysis and ion exchange chromatography and expression and purity were characterized using mass spectrometry and amino acid analysis. Both ELP1-CSBDs were successfully expressed using these methods and ELP1-CSBD1 was produced to high purities. ELP1-CSBD1 was able to undergo coacervation *in vitro,* suggesting that ELP1-CSBD1 is able to self-assemble in a manner similar to native elastin. In the presence of the glycosaminoglycan chondroitin sulphate (CS), the temperature of coacervation of ELP1-CSBD1 is increased, the rate and extent of coacervation is decreased, and aggregates remain in solution even at higher temperatures. The influence of heparin was also explored as previous studies indicated that the CS binding domains were shown to also bind to heparin. Studies completed in the presence of heparin showed that there were no

significant changes to the coacervation characteristics of ELP1-CSBD1. It is anticipated that when combined with CS, ELP1-CSBD1 will gel, forming a basis for an intimal/medial layer of a TEBV that will modulate SMC response and increase graft integrity.

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List of Abbreviations

A	alanine
bp	base pairs
$CaCl_2$	calcium chloride
CS	chondroitin sulphate
C4S	chondroitin-4-sulphate
C6S	chondroitin-6-sulphate
CNBr	cyanogen bromide
CSBD	chondroitin sulphate binding domain
EBP	elastin binding protein
EC	endothelial cell
ECM	extracellular matrix
EGF	endothelial growth factor
ELP	elastin-like polypeptide
ELP-CSBD	elastin-like polypeptide containing a chondroitin sulphate binding domain
ESI-MS	electrospray ionization mass spectroscopy
G	glycine
GAG	glycosaminoglycan
GalNAc	N-acetylgalactosamine
GRAVY	grand average of hydropathy
GST	glutathione-S-transferase
HA	hyaluronan
HS	heparan sulphate
IPTG	isopropyl-K-D-thiogalactopyranoside
K	lysine
LB	lysogeny broth
LPS	lipopolysaccharides
m/z	mass to charge ratio
MALDI	matrix assisted laser desorption/ionization
MS	mass spectroscopy
MW	molecular weight
MWCO	molecular weight cut off

NaCl	sodium chloride (salt)
OD	optical density
P	proline
PAD	peripheral arterial disease
PAOD	peripheral artery occlusive disease
PGA	polyglycolic acid
PLA	polylactic acid
PU	polyurethane
PVD	peripheral vascular disease
PTFE	polytetrafluoroethylene
SEM	scanning electron microscopy
SMC	smooth muscle cell
SOC	super optimal broth with catabolite repression
T_c	coacervation temperature
TEBV	tissue engineered blood vessel
THPP	β-[tris(hydroxymethyl)phosphino]-propionic acid
TOF	time of flight
V	valine
VIC	valvular interstitial cell
VSMC	vascular smooth muscle cell
YT	yeast extract and tryptone

Chapter 1

Introduction

1.1 Rationale

Arterial replacement is now a common treatment for vascular disease with more than 1.4 million vascular bypass operations performed per year in the United States alone (McKee, 2003). However, the use of vascular grafts in applications where the vessel is less than 6mm in internal diameter remains a clinical challenge. Traditionally, synthetic polymers such as Dacron and expanded poly(tetrafluoroethylene) (e-PTFE) that are typically used in large-diameter vascular grafts have been unsuccessful in small-diameter environments due to thrombogenicity, aneurysm formation, and infection (Isenberg et al., 2006). Autologous grafts continue to be the current standard, but this method has its own challenges as many patients do not have a suitable replacement vessel available and the requirement of an additional surgical procedure is undesirable.

There are two main areas where small-diameter vascular grafts have significant therapeutic potential: to treat peripheral vascular disease (PVD) and as a method of vascular access in kidney dialysis. The prevalence of diabetes in a growing portion of the population and its associated peripheral vascular damage, particularly below the knee, has greatly increased the need for successful grafts in small-diameter applications. Since 1998, the number of Canadians diagnosed with diabetes has increased by 70% with almost 2.4 million Canadians, or approximately 6.8% of the population, living with the disease in 2009 (Public Heath Agency of Canada, 2011). Worldwide, this number exceeds 285 million, and is only expected to increase over the next ten years. While there are many complications associated with diabetes, damage to

peripheral blood vessels is a significant long-term problem that typically requires treatment using vascular grafts.

PVD is a circulation disorder involving the narrowing of peripheral arteries (not within the coronary vasculature or brain) that results in decreased blood flow to the body's extremities. Commonly referred to as peripheral arterial disease (PAD) or peripheral artery occlusive disease (PAOD), peripheral vascular disease is typically caused by a buildup of plaque in the inner walls of small-diameter arteries, a condition known as atherosclerosis (American Heart Association, http://www.heart.org/HEARTORG/Conditions/More/PeripheralArteryDisease/About-Peripheral-Artery-Disease-PAD_UCM_301301_Article.jsp, 2007). PAD is a common complication of diabetes, and it is currently estimated that one out of every three people with diabetes over the age of 50 has PAD (American Diabetes Association, http://www.diabetes.org/living-with-diabetes/complications/peripheral-arterial-disease.html, 2010).

Individuals with diabetes-associated PAD have an increased risk for the development of foot disorders that can lead to lower limb amputation (Frykberg, 2002). Diabetic foot ulcers are a common complication of diabetes and can often be attributed to the lack of blood flow to the lower extremities as a result of PAD. It can lead to severe infection and even gangrene of the foot, often requiring lower leg amputation. In 2009, Canadians with diabetes were approximately 20 times more likely to be hospitalized with lower limb amputations compared to those without the disease (Public Heath Agency of Canada, 2011), and approximately 85% of all diabetes-related lower limb amputations are preceded by foot ulcers (Pecoraro et al., 1990). People with PAD also have a higher chance of heart attack and stroke and are six times more likely to die within 10 years than those without PAD (Belch et al., 2003).

Synthetic grafts are also used as a method of access in kidney dialysis, a treatment option for end stage renal disease. More than 250,000 North Americans are estimated to be undergoing hemodialysis, and approximately 50% of those patients are using a graft for vascular access (Kidney Foundation of Canada, 2012; Weng & Berns, 2006). Arteriovenous grafts are the most commonly used as a method of vascular access, but these have many attendant risks including the development of stenosis and thrombosis that can lead to embolism or other complications. As a result, the need for a reliable vascular graft that is effective in small-diameter environments is of growing importance.

The increasing demand for small-diameter vascular grafts has exposed the need for alternative solutions to create a graft that can emulate the physical, mechanical, and biological functions of a natural blood vessel. Biomimetic strategies that use synthetic and/or biological-based materials are of increasing interest as an approach to address the inadequacies of current grafts. Specifically, tissue-engineered blood vessels (TEBVs) that have the ability to imitate natural blood vessels have significant therapeutic potential in applications where current synthetic and autologous grafts have failed. However it has proven to be difficult to design a TEBV that is able to meet vital structural and mechanical criteria while maintaining the necessary biological requirements. TEBVs based on natural polymers such as collagen and elastin have demonstrated potential but lack the mechanical properties, including strength and rigidity, necessary to support blood flow with burst pressures limited to approximately 225mmHg in collagen grafts (Song et al., 2011). In other TEBVs researchers have attempted to use only synthetic polymers like polyglycolic acid (PGA), but grafts based on synthetic materials alone – while inexpensive and highly tunable – are a substandard option as they can undergo rapid resportion that reduces

mechanical properties, have limited contractile response as they can lack elasticity, and can induce thrombus formation (Isenberg et al., 2006; Hoenig et al., 2005; Greisler et al., 1990).

One of the most common challenges associated with vascular graft failure is intimal hyperplasia. Arterial intimal hyperplasia is a typical wound healing response that involves the build up of smooth muscle cells (SMCs) in the intimal layer of the artery due to abnormal migration and proliferation (Clowes, 1993). This thickening of the inner arterial wall is a response to endothelial damage post-graft implantation and results in a narrowing of the lumen, which subsequently obstructs blood flow (Lemson et al., 2000). Intimal hyperplasia can be caused by loss of endothelium and damage to the arterial wall during graft implantation, compliance mismatch, and low blood flow (Clowes, 1993; Cox et al., 1991; Lemson et al., 2000; Isenberg et al., 2006). Although extensive research has been undertaken to improve vascular graft patency, intimal hyperplasia continues to be a leading cause of graft failure following implantation. Therefore, a graft that has the ability to regulate changes in SMC response while still meeting the necessary elastic requirements is highly desirable.

Elastin-like polypeptides (ELPs) are versatile polymers that possess several favorable properties of native elastin, an extracellular matrix (ECM) protein that is a fundamental component of the connective tissue that holds together and imparts elasticity to muscles, nerves, and blood vessels. The Woodhouse lab has successfully developed a family of ELPs that mimic the natural properties of human elastin (Bellingham et al., 2001). These qualities include high stability, the ability to self-assemble *in vitro*, and fibre forming capabilities. The Woodhouse ELPs have also exhibited low immunogenicity and low platelet activation, thus establishing them as good candidates for use in blood vessel applications (Woodhouse et al., 2004; Bellingham et al., 2001; Srokowski & Woodhouse, 2008). These recombinant ELP proteins hold potential for

use in a variety of biomedical applications as they may be designed with specific amino acid sequences to achieve unique biomaterial properties and functionality.

While their use in TEBVs is promising, ELPs alone may not be sufficient to address the main challenges associated with engineering a small-diameter vascular replacement. Chondroitin sulphate (CS) is a glycosaminoglycan (GAG) found in ECM of tissues. It has been shown to influence a variety of cell functions and may be capable of modulating smooth muscle cell response. Based on these properties, the incorporation of a CS binding domain into an ELP may result in the modulation of smooth muscle cell function, ultimately limiting intimal hyperplasia.

It is proposed that a combination of one of these ELPs developed in the Woodhouse lab (ELP1) and recognized CS binding domains (CSBDs) to form new block-copolymers have the potential to act as part of an intimal/medial component of a TEBV that can regulate SMC response.

1.2 Aims and Objectives

The overall objective of this project is to develop and characterize a new elastin-like polypeptide-chondroitin sulphate binding domain (ELP-CSBD) block copolymer that holds the potential to gel in the presence of CS. In evaluating this novel polypeptide, the specific aims of this study are:

1. To express (in an *E. coli* bioreactor system) and purify novel elastin-like polypeptides modified to incorporate chondroitin sulphate binding domains.
2. To characterize these novel polypeptides using mass spectrometry and amino acid analysis to assess purity and coacervation to evaluate self-assembly.
3. To investigate the influence of the GAG chondroitin sulphate on the self-assembly of the polypeptides.

Chapter 2

Literature Review

2.1 Blood Vessels

The human vascular system is comprised of a network of blood vessels that circulate blood to provide oxygen and nutrients to all parts of the body. There are three main types of blood vessels that range in size and function: arteries, veins, and capillaries. Peripheral arteries can be very small and can range from 3-6mm in internal diameter (Nerem & Seliktar, 2001; Cho et al., 2005). These small-diameter blood vessels are comprised of three different layers: the tunica intima, the tunica media, and the tunica adventitia. While all vessels have similar structural features, the thickness of each layer can vary depending on the type. For example, arteries (Figure 1) tend to have a thicker tunica media and narrower lumen than a vein, while capillaries have only an endothelium with no other tunica layers (Mescher, 2010). Furthermore, small-diameter vessels generally have thinner tunics than larger vessels, and lack the microvasculature associated with the tunica adventita of larger blood vessels (Mescher, 2010).

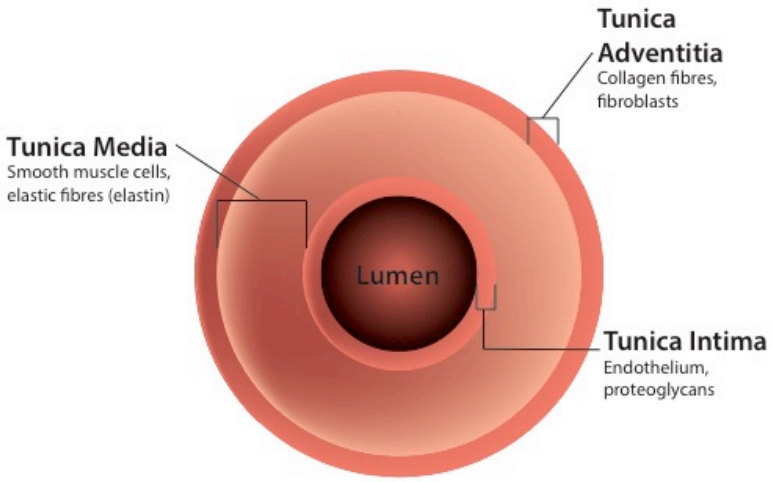


Figure 1: Cross-sectional view of the layers of a typical artery. Small-diameter vessels and arteries have thinner tunics, smaller luminal space, and lack the microvasculature found in the tunica adventitia of larger vessels.

The tunica intima is the innermost layer of the vessel and is in contact with the blood. It is made up of a thin lining of endothelial cells supported by a layer of connective tissue rich in basement membrane and matrix molecules such as proteoglycans, heparin sulphate, and laminin (Mescher, 2010). The tunica media is the thickest layer of the vessel and is primarily made up of smooth muscle cells (SMCs) and elastic tissue (McKee et al., 2003). The elastic tissue contains an abundance of elastin fibres and lamellae that allow the vessel to undergo series of extension and recoil, contributing to its ability to withstand the pressure fluctuations associated with blood circulation (Mescher, 2010; He et al, 2011). Finally, the tunica adventitia, the outermost layer, provides the vessel with rigidity and integrity. It is mainly comprised of loosely organized fibroblasts and collagenous and elastic extracellular matrix (ECM) (Mescher, 2010). It has clearly been established that the organization of the blood vessel is quite intricate, with each layer exhibiting its own structural and functional characteristics that are not easily replicated outside of the body.

2.1.1 Vascular Smooth Muscle Cells

Vascular smooth muscle cells (VSMCs) are highly specialized cells that are a main component of the vascular wall. They are found primarily in the vessel media, interspersed between the elastic lamina and surrounded by basement membrane (George & Johnson, 2012). VSMCs are responsible for a variety of different structural and physiological functions including contraction and regulation of blood vessel tone, blood pressure and flow. In addition, the mechanical properties of the vascular tissue are dependent on the volume and orientation of VSMCs in the media (Ebrahimi, 2009). The exposure of VSMCs to cyclic stress and strain, a result of blood flow through the vessel, is thought to be an important feature in the regulation of their function and helps to maintain the SMCs in a contractile state (Hipper & Isenberg, 2000; Sharifpoor et al., 2010). The majority of ECM components in the arterial wall including collagen, a major structural protein, are synthesized by VSMCs (Owens et al., 2004).

Morphogenesis and regulation of the blood vessel wall is highly dependent on the functioning of VSMCs. VSMCs are unique in that unlike other types of muscle cells that are terminally differentiated, they can undergo changes in phenotype during different stages of human development and even in adults as a result of alterations in their local environment (Owens, 1995). In a mature blood vessel under normal conditions, VSMCs exhibit a contractile phenotype and are in a non-proliferative and non-migratory phase, remaining in the medial layer of the vessel wall. This is regulated by the endothelium and ECM in the tunica intima that, when functioning normally, provides a barrier to circulating growth factors and inhibits increased SMC proliferation and migration (Marx et al., 2011). However, in response to vascular injury or vascular graft implantation, VSMCs can dedifferentiate and reenter the cell cycle to modify their phenotype and contribute to vascular repair (Owens et al., 2004; Rzucidlo et al., 2007).

Activation of surrounding growth factors and cytokines stimulate the VSMCs to migrate and proliferate into the vascular intima and participate in vascular remodeling (Marx et al., 2011). These alterations in SMC function, if not properly regulated, can lead to the development of vascular diseases such as atherosclerosis and hypertension.

2.2 Small-Diameter Vascular Grafts

The use of synthetic grafts in small-diameter environments has been limited by a variety of factors including thrombogenicity, aneurysm formation, progression of atherosclerotic disease, and infection. (Isenberg et al., 2006). While materials such as Dacron and expanded poly(tetrafluorethylene) (e-PTFE) have been highly successful in grafts larger than 6mm, when used in smaller applications they have been shown to be inadequate, with thrombosis rates greater than 40% after only 6 months (Niklason et al., 1999). Autologous vessels are the current standard in arterial replacement, however appropriate vessels can be difficult to obtain because of compliance mismatch or because they are simply not available. A human saphenous vein is typically used as the replacement vessel, and the subsequent surgical procedure required to obtain the vessel is unattractive as it is invasive and an unnecessary risk.

There are a variety of challenges when it comes to designing a vascular graft for use in a small-diameter environment. Compared to larger vessels, small-diameter vessels have lower blood flow velocities and higher resistance to fluid flow (L'Heureux et al., 1998, Isenberg et al., 2006). This combination increases the chance of thrombus formation, and it has proven to be difficult to find suitable materials to mitigate this risk. Researchers have shown that endothelial cells (ECs) in the vasculature play a role as part of an anticoagulant surface as they actively inhibit thrombosis and additionally limit hyperplasia through the regulation of SMC proliferation and migration (Heyligers et al., 2005; Gimbrone et al., 1990). The lack of an endothelial layer has

been certified as a main reason why synthetic small-diameter grafts have had limited success (Heyligers et al., 2005; Avci-Adali et al., 2008).

2.2.1 Intimal Hyperplasia

One of the most common challenges associated with vascular graft failure is intimal hyperplasia, a universal response to vessel injury involving the thickening of the intimal layer due to abnormal VSMC migration and proliferation (Clowes, 1993).

Following vascular injury, different cells in the arterial intima, including endothelial cells, release chemical mediators such as growth factors, cytokines, proteins, and proteoglycans that induce vascular wall repair mechanisms (Marx et al., 1995; Charboneau et al., 2011). This includes the stimulation of the phenotypic change of VSMCs from their stationary, contractile state to an active phase, enhancing proliferation and migration of the cells inward from the media to the intima (Willis et al., 2004). Once in the intima, the VSMCs continue to proliferate and begin to produce ECM components in order to repair the vascular wall. This increase in the number of SMCs and extracellular matrix deposition in the intima contributes to the thickening of the inner arterial wall and results in stenosis of the vessel, subsequently obstructing blood flow (Lemson et al., 2000).

Intimal hyperplasia can be caused by a variety of factors. Typically, hyperplasia is promoted by the loss of endothelium and damage to the arterial wall during vascular graft implantation (Clowes, 1993). Endothelial cells undergo apoptosis, releasing mediators that signal for the proliferation and migration of VSMCs into the intimal layer. In addition to graft implantation, compliance mismatch between the graft and the vessel can also influence endothelial function and the formation of intimal hyperplasia (Cox et al., 1991; Lemson et al., 2000; Isenberg et al., 2006). While normal vessels respond to low blood flow by constricting,

rigid grafts based on synthetic polymers cannot properly mimic this function (Clowes, 1993). To compensate, surrounding endothelial cells increase the intimal thickening to maintain the appropriate shear stress. In general, decreased blood flow has been thought to influence intimal hyperplasia as it promotes thickening of the intima and endothelial cell apoptosis in response to reduced wall shear stress (Sho et al., 2001). Overall, abnormal VSMC proliferation and migration, particularly after vascular graft implantation, play a key role in the development of intimal hyperplasia.

Although extensive research has looked into improving vascular graft patency, intimal hyperplasia continues to be a leading cause of graft failure. Current therapies involving coronary grafts have focused on specifically targeting and inhibiting VSMC migration and proliferation by creating drug-eluting grafts that release antiproliferative drugs such as rapamycin (Poon et al., 1996; Marx et al., 1995). While this approach has been successful at reducing the proliferation of VSMCs, drug-eluting stents have been investigated mainly in coronary applications as opposed to peripheral applications. In addition, drug-eluting stents have been shown to increase the risk of thrombosis following implantation and there are concerns about the safety of this therapy (Maisel & Laskey, 2007; Balcells et al., 2010). Recent research has also looked into the selective control of VSMC progenitors in the vessel wall, however this is a controversial therapy and further studies elucidating the role of these progenitor cells in vascular disease is required (Marx et al., 2011).

2.3 Tissue Engineered Blood Vessels

Tissue engineered blood vessels (TEBVs) are a promising approach to circumvent the problems encountered with current small-diameter grafts through the combination of natural and/or synthetic materials. Basic criteria that have been proposed for the design of biocompatible

TEBVs include non-thrombogenicity, non-immunogenicity, resistance to infection, and the ability to induce an acceptable healing response (Isenberg et al., 2006). The development of TEBVs that have the ability to meet these criteria and successfully mimic the physical, mechanical, and biological functions of natural blood vessels, however, has proven to be more difficult to develop than anticipated.

Different strategies have already been used to engineer TEBVs, mainly focusing on the use of either natural or synthetic materials. Synthetic polymers have been unsuccessful in small-diameter applications as some, including polyglycolic acid (PGA) and polylactic acid (PLA), are prone to biodegradation in the body, leading to loss of mechanical properties in the graft. Alternatively, completely biologically-based TEBVs have been proposed, but these have been shown to lack the structural and mechanical properties required of a small-diameter vessel. Studies have shown that although they meet the biological criteria, decellularized vessels composed of only ECM materials are not always mechanically compatible in graft applications and can sometimes shrink, resulting in reduced tensile strength (Isenberg et al., 2006). L'Heureux et al. (1998, 2006) have succeeded in engineering TEBVs based solely on the use of human cells without any synthetic materials, but clinical studies have yet to be performed and efficacy following implantation is not yet known.

More recent focus has been on biomimetic strategies that combine synthetic and biological-based materials to form artificial scaffolds that will imitate the basic behavior and functions of native vasculature. Researchers have been investigating the use of tubular constructs consisting of synthetic polymer scaffolds and ECM materials (Wang, 2007). This innovative method has potential in small-diameter applications as the constructs can be designed to mimic the structural and functional elements of native vessels. In addition, these engineered vessels

would have the potential to repair and remodel the functional tissue of the natural blood vessel, acting as a temporary scaffold that is able to support restoration by inducing cell response.

Much of the research into biologically-based small-diameter blood vessel grafts has focused on the inclusion of arterial cells such as endothelial cells (ECs) and SMCs. Many approaches involve grafts created by culturing VSMCs onto tubular scaffolds of biodegradable polymers (Greenwald & Berry, 2000). Cell seeding is a relatively recent technique, with investigators seeding the lumen of synthetic grafts with ECs (Noishiki, 1996; Teebken & Heverich, 2002) or SMCs (He et al., 2011; Dahan et al., 2012), both have which been found to accelerate the formation of an endothelial lining on the luminal surface of synthetic grafts. Although in theory this is a promising approach, seeding cells onto artificial surfaces has been faced with a number of obstacles including sloughing of the seeded layer, chronic inflammation and other immunogenic responses, and unwanted phenotypic changes (Heyligers et al., 2005; Yokota et al., 2008; Ballermann, 1998). It is believed that the presence of these cells would improve the patency of small-diameter grafts as they would establish a compatible lining on the blood contacting surface and would be less likely to induce an abnormal healing response. However, the inclusion of these cells has proven to be difficult, and the search continues for a TEBV that has the ability to successfully establish a compatible intimal layer without stimulating abnormal proliferation and migration of SMCs.

It is widely agreed that the ideal biological graft should be mechanically durable, elastic, degradable, biocompatible, and the inner surface must be hemocompatible (Niklason et al., 1999; Baguneid et al., 2006; Song et al., 2011). While many researchers are focusing on attempts to imitate an endothelial-like layer, the Woodhouse group believes that a successful biomimetic graft will need to be multilayered and have the ability to regulate changes in SMC response while

meeting the necessary elastic requirements to support blood flow. In this work, the focus is on the use of polypeptide-based biomaterials in the development of a medial material that will interface well with an intimal layer and modulate SMC response.

2.4 Elastin

The elastin protein (elastin) is an insoluble ECM protein that is a major component of elastic fibres. It is found in tissues that require extensibility and elastic recoil including blood vessels, skin, lung, bladder and ligaments (Vrhovski et al., 1997; Bellingham, 2001; MacEwan & Chilkoti, 2009). Besides imparting elastic properties to elastic fibres, elastin is important in maintaining mechanical and structural integrity of tissues and has also been found to play a role in cell signaling and proliferation (Yeo et al., 2011; Brooke et al., 2003; Arribas et al., 2006). Unlike other ECM proteins, elastin synthesis occurs only in early development and little to no turnover happens following maturation (Davis, 1993). However, elastin is extremely durable and is able to undergo billions of cycles of stretching and relaxation without failure (Urry et al., 1995).

Elastin is found in high concentrations in the ECM of major vascular arteries and can contribute to up to 50% of a vessel's dry weight (Parks et al., 1993). It is synthesized and secreted by SMCs in the arterial media and undergoes crosslinking by lysyl oxidase in the extracellular space (Kagan & Trackman, 1991). In the medial layer of the vessel wall, elastic fibres are arranged in a network of concentric lamellae that are physically connected to a concentric ring of SMCs, forming the lamellar unit of the arterial wall (Arribas et al., 2006). As such, the proper functioning of elastin and correct elastic fibre assembly is critical in maintaining suitable blood flow throughout the body.

2.4.1 Structure

Tropoelastin is the soluble precursor to elastin. It contains a repeating domain structure that consists of alternating hydrophobic and crosslinking regions. The hydrophobic domains of tropoelastin are rich in repetitive blocks containing amino acids proline (P), valine (V), and glycine (G), and are believed to be responsible for the self-aggregation and elastomeric properties of elastin. Alternatively, the crosslinking domains contain predominantly alanine (A) and lysine (K) and are thought to be involved in the stabilization of elastin *in vivo* through the formation of crosslinks. (Vrhovski & Weiss, 1998; Bellingham & Keeley, 2004; Rodgers & Weiss, 2005).

Elastin is highly hydrophobic for a protein in that approximately 75% of the entire sequence is comprised of just G, V, A, and P, which are often found in an arrangement of PGVGVA. This sequence repeats seven times in tandem and is coded in exon 24 of the elastin gene (Szabó et al., 1999; Bellingham & Keeley, 2004). An additional repetitive sequence is found in the positioning of the crosslinking alanine and lysine residues, which are typically found in KxxK or KxxxK arrangements (Miao et al, 2005). The hydrophobic domains have been shown to have a highly flexible structure consisting of a predominately β-structure, specifically type-II β-turns, while the crosslinking domains are believed to form α-helical segments (Debelle et al., 1992; Miao et al., 2005; Tamburro et al., 2003; Bochicchio et al., 2008).

It is believed that tropoelastin is escorted through the cell to the surface in preparation for assembly into elastic fibres by a molecular chaperone termed the elastin binding protein (EBP) (Hinek & Rabinovitch, 1994; Hinek et al., 1998). Once deposited in the ECM, lysyl oxidase deaminates the lysine residues found in the crosslinking regions to enable covalent crosslinking to occur, thus forming a stable elastin matrix (Bellingham, 2001). It is the α-helical formations of the crosslinking domains that are believed to bring the lysine residues to the same side of the

helix, giving rise to the formation of intermolecular crosslinks between them (Debelle et al., 1992; Vrhovski & Weiss, 1998). The distinctive structure of elastin plays an important role in its ability to assemble into an elastic polymeric network.

2.4.2 Properties

Elastin has many unique physical, mechanical, and biological properties that make it a protein of great interest for clinical applications. The ability of elastin to assemble and form fibres makes it an important structural component of many tissues. The mechanical properties of elastin are essential in the functioning of blood vessels, allowing them to expand and contract in accordance with the pulsations of blood flow. Additionally, once crosslinked into a stable polymer, elastin becomes insoluble and is almost completely resistant to proteolytic cleavage (Vrhovski & Weiss, 1998). This also enables tissues containing elastin to be subjected to high pressures with little to no damaging effects.

In addition to its resilience and tensile properties, elastin has many important biological features including low platelet activation and low immunogenicity. Research has shown that elastin is not a strong agonist of platelet activation and there is limited platelet adhesion to isolated elastin (Baumgartner, 1976; Spaet & Erichson, 1966; Barnes & MacIntyre, 1979). Recent studies by Simionescu et al. (2006) have even shown that arterial scaffolds made of pure elastin exhibit low platelet reactivity in bypass grafts in a rabbit model. However, while it is well established that elastin has relatively low reactivity to platelets, the reasons for this have yet to be elucidated. This property of low platelet activation and adhesion means that when elastin is in contact with blood, it does not have a high tendency to produce a thrombus, which is an important characteristic for a component of an engineered blood vessel. Investigators have also shown that elastin has an effect on both EC and SMC response. Karnik et al. (2003) were able to show that

elastin regulates SMC proliferation and migration in culture, and reduces the proliferative response in an injured artery. Additionally, other studies have shown that elastin in the internal lamina of blood vessels inhibits SMC migration (Grande et al., 1987; Ooyama et al., 1987).

In many of the current vascular grafts that have been proposed, elastin is not a major component, and it is speculated that the lack of elasticity in grafts as a result of the absence of elastin may be a major reason for graft failure in small-diameter applications (Song et al., 2011).

2.5 Elastin-Like Polypeptides (ELPs)

Elastin-like polypeptides (ELPs) are recombinant proteins derived from the elastin gene. Those considered for biological applications are generally comprised of repeat units of a series of exons from the elastin gene, and are synthesized using recombinant DNA technology. Most ELPs consist of repeats of the sequence VPGXG where X, the guest residue, is any amino acid other than proline (MacEwan & Chilkoti, 2009). These peptides are typically expressed using a recombinant bacterial system such as *E. coli*. ELPs have been shown to have analogous properties to native tropoelastin, including similar self-assembly abilities (Cox et al., 1974; Urry, 1982; Urry, 1995; Vrhovski et al., 1997; Bellingham et al., 2001; Miao et al., Osborne et al., 2007) and chemotactic properties. ELPs are therefore an attractive material for use in biomedical applications, and can even be designed with specific amino acid sequences to achieve unique properties and functionality.

The Woodhouse laboratory has expressed, produced, purified and extensively characterized a family of recombinant ELPs that mimic the alternating domain structure of native tropoelastin (Bellingham et al., 2001; Keeley et al., 2002; Bellingham et al., 2003; Osborne et al., 2007). Each of the peptides contains at least one crosslinking domain flanked by two hydrophobic domains. The hydrophobic regions are coded for by exons 20 and 24 of the human

elastin gene and the crosslinking regions are coded for by exons 21 and 23. The nomenclature used for each polypeptide is derived from the exons that code for the crosslinking regions in the ELPs; for example, ELP-20-24 (ELP1) contains one crosslinking domain flanked by two hydrophobic regions, while ELP-20-24-24-24-24 (ELP4) contains four crosslinking domains flanked by five hydrophobic domains. A schematic diagram of the ELPs is shown in Figure 2.

Figure 2: Expression constructs of the Woodhouse family of ELPs. All peptides were generated as glutathione S-transferase (GST) fusion proteins. The GST cleavage site is indicated for ELP1 and is the same for the other constructs. Polypeptide sequences coded for by each of the exons are also shown. Crosslinking exons are in pink, while hydrophobic exons are in blue. Note the repeating PGVGVA sequence in Exon 24. Adapted from Bellingham et al., 2001 and Bellingham et al., 2003.

This family of recombinant ELPs have been investigated as a potential non-thrombogenic coating on synthetic materials and it was shown that coated surfaces were rendered non-thrombogenic and possibly non-coagulating, indicating that platelet activation was decreased *in vitro* (Woodhouse et al., 2004). Additionally, when a catheter was coated with one of these ELPs and placed in a rabbit model, the patency and efficacy of the catheter was increased (Woodhouse et al., 2004).

2.6 Self-Aggregation of the Elastin Protein

One of the most intriguing and distinctive properties of elastin is its ability to self-assemble. This ability of tropoelastin to spontaneously align and form fibrillar structures is thought to be an essential step in the formation of elastin and therefore an imperative part of elastic fibre formation. Self-assembly that occurs *in vitro* is termed coacervation.

2.6.1 Coacervation

Coacervation is a temperature-induced phase separation in which a protein will form an aggregate upon an increase in temperature, and will separate from the bulk solution as a second phase (Urry, 1982; Bellingham, 2001). The process of coacervation has been described for both the native elastin protein and recombinant elastin polypeptides (Cox et al., 1974; Urry, 1982; Urry, 1995; Vrhovski et al., 1997; Urry, 1999; Miao et al., 2003) and previous work by Bellingham et al. (2001) has showed that the Woodhouse family of ELPs also coacervate.

2.6.1.1 Mechanism of Coacervation

While the coacervation of elastin has been well established for many years, the mechanism of this behaviour has yet to be fully understood. This phenomenon is usually explained in terms of the effects of water on the elastin or elastin-like molecule. It is speculated that the mechanism of coacervation involves a phase transition where the hydrophobic groups of elastin, initially hydrated in water, become folded and assembled with an increase in temperature (Urry, 1999).

In solution, water molecules arrange themselves around the elastin molecules. Due to the inability of the water to interact with the hydrophobic components of elastin, the water molecules form hydrogen bonds with each other resulting in a lattice of pentagonal structures surrounding the elastin. This “clathrate water structure” is believed to shield the elastin and help to keep the

molecule in an extended shape. With an increase in solution temperature, the hydrogen bonds in the clathrate become disrupted and its structure becomes disordered. Unhindered by the arrangement of water surrounding it, the hydrophobic domains of the elastin are able to interact and the protein can assume a folded shape, also known as a coacervate (Urry, 1995).

The coacervation of elastin can also be referred to as an inverse temperature transition because the structure of the elastin molecule becomes highly ordered with an increase in temperature, unlike most proteins that become less ordered and denature under higher temperatures (Urry, 1995). Coacervation is an entropic phenomenon in that the increase in the disorder of the hydrating water molecules is greater than the increase in the order of the chain, hence increasing the total entropy of the system (Urry, 1995; Cirulis & Keeley, 2010).

2.6.1.2 Structure of the Coacervate

Studies have shown that prior to coacervation, tropoelastin exists as small monomers in globular arrangements (Bressan et al., 1986). Structurally, the coacervate that is formed as a result of coacervation is typically seen as a fibrous arrangement (Cox et al., 1974) and fibrillar structures have been shown to form from coacervates of tropoelastin (Bressan et al., 1983). This configuration is often attributed to the folding of the helical structure of the elastin, which promotes interactions between side chains and aligns its hydrophobic domains. The temperature effects on the interactions between the hydrophobic domains and water, as previously described, are the main driving force behind the folding or extension of the spiral.

Similar to native elastin, ELP chains also start in an extended state and then fold into a helical conformation as temperature is increased (Urry, 1995). Keeley et al. (2002) determined that only recombinant ELP proteins that contained hydrophobic domains had the ability to coacervate, confirming the role of the hydrophobic residues in the formation of the aggregate.

2.6.1.3 Coacervation Temperature

The temperature at which coacervation occurs is taken as a measure of the propensity of elastin to self-aggregate (Urry, 1982). This temperature is dependent on a variety of factors including pH, ionic strength, protein concentration, and protein hydrophobicity.

The pH of the solution has a significant effect on the coacervation temperature; studies have shown that increasing the pH lowers the temperature of coacervation (Vrhovski et al., 1997; Wu et al., 1999; Bellingham et al., 2001). It is thought that changes in pH influence the balance of polar and nonpolar groups on the protein, which in turn has an effect on the interactions between the protein and solvent water, possibly weakening the hydrophobic associations required to drive self-aggregation (Yeo et al., 2011). Coacervation has been shown to maximally occur at the isoelectric point of the protein or polypeptide (Partridge et al., 1980; Hinek & Rabinovitch, 1994).

Ionic strength, typically measured by salt concentration, has a similar effect on coacervation temperature in that increasing the ionic strength of the buffer solution decreases the temperature of coacervation. This relationship can also be attributed to the disruption of protein-water interactions. An increase in ionic strength means an increase in salt ions, and these ions induce the formation of hydration shells around them. Water preferentially goes into these arrangements, subsequently reducing the strength of the clathrate structures surrounding the elastin molecules. It is thus easier to destabilize these clathrate shields and therefore coacervation is able to occur at lower temperatures (Urry, 1995; Vrhovski et al., 1997)

Increasing the concentration of elastin or elastin polypeptide has also been shown to lower the coacervation temperature as well as increase the rate of coacervation (Vrhovski et al., 1997, Bellingham et al., 2001, Toonkool et al., 2001). This can typically be attributed to an

increased number of polypeptide molecules that are able to interact with each other, as the formation of water clathrate shields would be more difficult in higher concentrations of peptide.

Finally, the arrangement and frequency of hydrophobic and hydrophilic domains in elastin has a significant effect on self-assembly and the temperature at which it takes place. There is an inverse relationship between coacervation temperature and mean hydropathy of a peptide, as elastin peptides containing less hydrophobic domains have been shown to coacervate at higher temperatures (Bellingham et al., 2001; Miao et al., 2003). Additionally, while the presence of hydrophobic domains is a necessity for self-aggregation to occur, peptides based only on hydrophobic domains have a lower propensity for self-aggregation, and therefore higher coacervation temperature, compared to peptides with both hydrophobic and hydrophilic domains (Miao et al., 2003). Therefore, the presence of hydrophilic domains helps determine the ability of elastin to coacervate.

Each of these phenomena affecting coacervation temperature has been shown for native tropoelastin as well as different recombinant versions of ELPs (Vrhovski et al., 1997; Bellingham et al., 2001; Cirulis & Keeley, 2010). Full-length human tropoelastin has been shown to optimally coacervate at 37°C under physiological conditions that mimic those found in the ECM, suggesting that coacervation occurs rapidly in the extracellular space (Vrhovski & Weiss, 1998).

2.7 Glycosaminoglycans

Glycosaminoglycans (GAGs) are highly charged polysaccharides that are found in abundance in the ECM. They are long, linear chains of repeating disaccharide units that can be found covalently linked to proteins, forming proteoglycans (Lindahl & Hook, 1978; Lodish et al., 2003). One or both of the sugar units contain at least one anionic group, which imparts a strong negative charge to the GAG chain (Lodish et al., 2003). There are four main classes of GAGs,

classified based on the nature of the disaccharide units and the extent of sulfation: chondroitin sulphate (CS), heparin sulphate (HS), hyaluronan (HA), and keratin sulphate (KS) (Ferdous & Grande-Allen, 2007).

Generally known as important structural components, GAGs have also been shown to have an effect on many cellular functions including attachment, motility, differentiation, and proliferation (Orlidge & D'Amore, 1986). GAGs regulate protein activity through their ability to bind to and modulate a variety of protein families such as growth factors, adhesion molecules, and ECM components (Fromm et al., 1997; Butterfield et al., 2010). Certain GAGs even play a role in the blood coagulation pathway and have antithrombotic effects (Bourin & Lindahl, 1993).

2.7.1 Chondroitin Sulphate

Chondroitin sulphate (CS) is the most prevalent glycosaminoglycan. It is a linear polysaccharide composed of the repeating disaccharide units containing N-acetylgalactosamine (GalNAc) and glucuronic acid joined by β-linkages (Lodish et al., 2003). The two most abundant types of CS are chondroitin 4-sulphate (C4S) and chondroitin 6-sulphate (C6S), which are named based on the sulphation of the hydroxyls at the 4 and 6 positions of the GalNAc, respectively (Volpi, 2006). The chemical structures of these forms of CS are shown in Figure 3. CS is typically found in the ECM of cartilage, the lining of blood vessels, the surface of the bladder, and the connective tissue that holds together muscles and nerves.

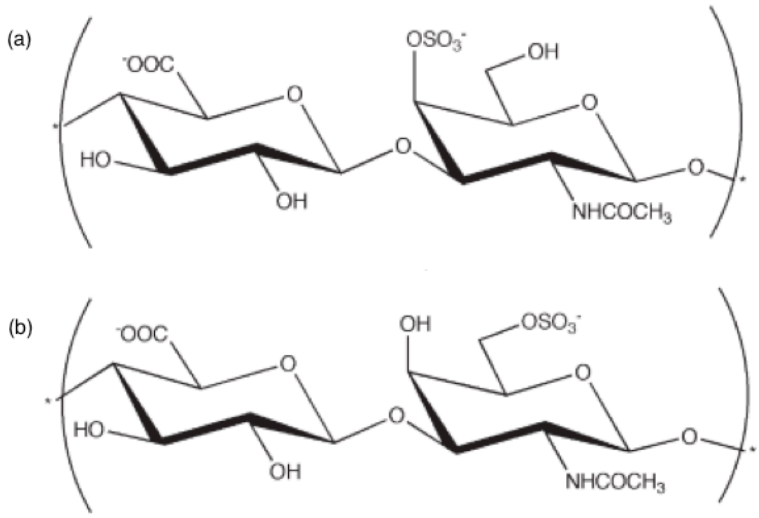


Figure 3: The repeating disaccharide units of two configurations of chondroitin sulphate (CS), C4S (a) and C6S (b). The CS monomer is comprised of repeating glucuronic acid and N-acetylgalactosamine units. C4S is sulphated at the C4 position of GalNAc while C6S is sulphated at the C6 position. Reprinted and adapted with permission from Butterfield et al., Identification and Sequence Composition Characterization of Chondroitin Sulfate-Binding Peptides through Peptide Array Screening. Biochemistry (2010) Volume 49 Issue 7. Copyright 2010 American Chemical Society.

CS interacts with various molecules in the ECM, including elastin, to influence organization and cell behaviour (Butterfield et al., 2010). CS has also been found to accumulate in the walls of atherosclerotic or wounded blood vessels, specifically in the medial layer (Hinek et al., 1992; Papakonstantinou et al., 1997). Some researchers have speculated that this increase in CS may trigger impaired assembly of elastin fibers (Hinek et al., 1991).

Paracrine mediators stimulated by apoptotic endothelial cells during vascular injury are known to release CS in an attempt to inhibit neighboring cell apoptosis and enhance healing of the intimal wall (Raymond et al., 2004). Using a fucosylated chondroitin sulphate, Tapon-Bretaudière et al. (2000) demonstrated the effect of CS on vascular cells, including the inhibition of SMC proliferation as well as enhancement of endothelium wound repair. Work done by Charbonneau et al. (2007) demonstrated that CS actually triggers several key mechanisms of

vascular repair and increases smooth muscle cell adhesion to certain polymeric grafts. In addition, it was shown that a potential graft coating that combines CS and endothelial growth factor (EGF) increases VSMC adhesion, growth, and resistance to apoptosis in serum-free medium (Charbonneau et al., 2011).

2.8 ELP-CSBDs

It is apparent that ECM components, including proteins and GAGs, have potential to provide a natural, biocompatible material that is able to mimic properties of natural tissues. Based on the properties of both elastin/elastin-like polypeptides and chondroitin sulphate, the Woodhouse Lab has proposed the incorporation of CS binding domains into ELPs for the development of a material that could be used in biocompatible TEBVs. It is anticipated that exposure of the proposed ELP-CSBD polymers to CS will elicit the formation of physical cross-links and will result in gelation, enhancing the structural stability of the material.

Hydrogels made of polypeptide-based materials are an attractive option for tissue engineering applications as they can be easily manipulated to exhibit different physical and mechanical properties, are highly biocompatible, can replicate natural tissue environments, have inherent cellular interaction capabilities, and can be used as a vehicle for cell and drug delivery (Lim et al., 2007; Burdick & Prestwich, 2011: Jonker et al., 2012). Recent studies into synthetic polypeptide-based hydrogels have been focused on ELP-based gels. Lim et al. (2007) have suggested that β-[tris(hydroxymethyl)phosphino]-propionic acid (THPP)-crosslinked ELP hydrogels have the potential to support tissue regeneration in a load-bearing environment. Chemically-crosslinked three-dimensional ELP-based hydrogel scaffolds have also been developed (Martin et al., 2009). When cultured with human umbilical vein endothelial cells, these scaffolds allowed for cell adhesion onto the surface and integration into the inner layers of

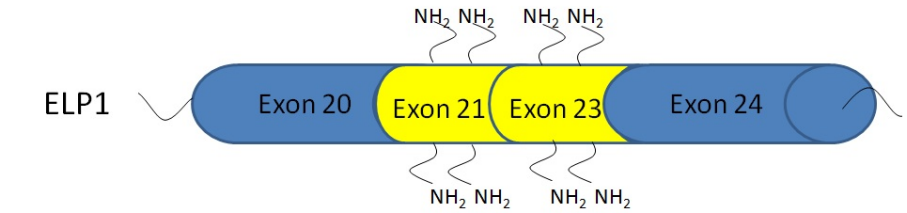
the gel, suggesting that this type of ELP hydrogel has potential for use in tissue engineering as an artificial ECM (Martin et al., 2009).

While a number of CS-based hydrogels have been developed (Kirker et al., 2002; Li et al., 2003; Gilbert et al., 2004; Conovaloff & Panitch, 2011), there has been limited investigation of these gels in vascular environments. Synthetic hydrogels made from crosslinked hyaluronan (HA) have been investigated in these applications, and have been shown to enhance natural biological repair processes when implanted in the body, and hold promise in cell delivery and expansion (Burdick & Prestwich, 2011). HA gels have been specifically looked at as substitutes for heart valves due to the presence of HA in native valves, and valvular interstitial cells (VICs) have been shown to adhere to and proliferate on the gels (Masters et al., 2004). In addition, VICs cultured on these surfaces displayed increased production of ECM proteins, indicating that HA-based gels may be able to stimulate heart valve tissue formation (Masters et al., 2004). It can be anticipated then, that in similar applications, TEBVs containing ELP-CS-based gels will show comparable results in terms of SMC proliferation and ECM production as elastin and chondroitin sulphate are components of the arterial wall and/or present during vascular wall repair.

2.8.1 Structure and Sequence

The sequence of the original ELPs designed by the Woodhouse Lab (Bellingham et al., 2001; Keeley et al., 2002; Bellingham et al., 2003) will be modified by removing the conventional crosslinking domains and replacing them with domains that will bind to chondroitin sulphate. Panitch et al. have previously identified amino acid sequences that are critical for CS binding (Personal communication, Jeong et al., 2008). Investigations probing CS-peptide interactions identified three different peptide sequences that were good binders to chondroitin sulphate using a combinatorial microarray study (Jeong et al., 2008). The ELP-CSBD sequence

compositions that we have developed in the Woodhouse Lab are based on these findings (Figure 4).



Exon 20: PGFGVGVGGIPGVAGVPGVGGVPGVGGVPGVGISP

Exon 21: EAQAAAAAKAAKY

Exon 23: GVGTPAAAAAKAAAKAAQFG

Exon 24: LVPGVGVAPGVGVAPGVGVAPGVGLAPGVGVAPGVGVAPGVGVAPGIGP

CSBD1: IKFIHRKYRKHG

CSBD2: RVFKRYKRWLHG

CSBD3: WRFRKFPKWRHG

Figure 4: Schematic of the ELP1 and ELP1-CSBD structures. The corresponding amino acid sequences are shown for exons 20,21,23 and 24. In the CSBD structures, exons 21 and 23 are replaced with the CSBD1, CSBD2, or CSBD3 sequence.

The Woodhouse elastin-like polypeptide ELP1 will be used as the backbone for the ELP-CSBD constructs. This study will focus on investigating the binding sequences of CSBD1 and CSBD2, but it is important to note that one other sequence, CSBD3 (shown in Figure 5) has the potential to also be used as a binding site in future work. Previous work using polymers containing heparin-binding domains have shown their ability to spontaneously form gels upon exposure to heparin (Seal & Panitch, 2003; Jeong & Panitch, 2009), supporting the theory that these ELP1-CSBDs will also gel in the presence of CS.

2.8.2 Potential Uses

The main objective of the development of these ELP1-CSBDs is to use them in combination with a biodegradable polyurethane (PU) that has already been designed in the Woodhouse laboratory (Skarja & Woodhouse, 2008; Skarja & Woodhouse, 2001; Parrag & Woodhouse, 2010) to form a composite material with the potential as an intimal/medial layer of a TEBV. Prior to electrospinning of the ELP1-CSBD and PU materials into a polymer scaffold, it is anticipated that exposure to CS will cause the CSBD to fixate CS in the composite scaffold, which will help to form physical crosslinks with the PU.

As previously discussed, a variety of research has already focused on the use of elastin-like polypeptides in biomaterials and specifically in engineered vascular grafts. Grafts that contain elastin have been shown to succeed at a high rate (L'Heureux et al., 1998), while the absence of elastin in the design of other vessel grafts has been seen as a major limitation and a reason behind graft failure (Weinberg et al., 1986). It is expected that the addition of CS to the ELP1-CSBDs will enhance the mechanical and biological properties of a potential graft versus one that is based solely on elastin. It is assumed that combining CSBD material and CS will result in gelation and will therefore prevent delamination and failure of the composite material. As previously outlined, CS has been shown to trigger several key mechanisms of vascular repair, which would be a beneficial characteristic of an intimal/medial component. Recent work has even shown that a potential graft coating that combines CS and endothelial growth factor (EGF) increases VSMC adhesion, growth, and resistance to apoptosis in serum-free medium (Charbonneau et al., 2011).

To further justify the rationale behind the combination of elastin-based materials with CS, there have been many studies indicating the cooperative behaviour between elastin and

chondroitin sulphate in the body. Hinek et al. (1992) have shown that CS facilitates cell migration and the production of fibronectin by SMCs in the presence of elastin. This suggests that an ELP-based graft that is combined with CS could potentially promote migration of tissue cells that are surrounding the implanted graft and thus allow for incorporation of the TEBV into the body. It has also been proposed that tropoelastin is encouraged to participate in elastogenesis by association with GAGs (Wu et al., 1999), which could be beneficial if tissue healing around the vessel is desired. Furthermore, chondroitin sulphates are closely associated with the developing elastic fibre (Sherrat et al., 1997) and occur at sites of elastin deposition in the healing of full-thickness wounds (Lamme et al., 1996). As previously discussed, ELPs on their own exhibit poor mechanical properties. Due to its high water solubility, pure chondroitin sulphate gels have also shown reduced mechanical properties compared to other biomaterials (Charbonneau et al., 2007), so therefore the combination of both could enhance mechanical resistance, which is especially important in vascular grafts that are exposed to high levels of pressure due to blood flow.

The rationale behind the combination of the ELP-CSBD/CS material with a biodegradable PU is to increase the mechanical properties of the graft. While it has been established that the incorporation of elastin is important to the success of a small-diameter TEBV, a major limitation of using a completely elastin-based graft is that it lacks the necessary mechanical stability, which is only further enhanced by the loss of mechanical properties that occurs when proteins are electropsun (Matthews et al., 2002; Wnek et al., 2003). Therefore, in order to overcome this issue, a biodegradable, segmented PU with elastic characteristics will be added for additional mechanical support. It is anticipated that this PU will also have the ability to

undergo degradation in the presence of matrix metalloproteinases (MMPs), proteases that are important in cell migration, tissue healing, and remodeling (Sprague & Khalil, 2009).

ELP-CSBD polypeptides could also be used in a variety of other applications. Not only would a TEBV made of this material have significant potential in the peripheral vasculature, but it could also be used in arteriovenous access for kidney dialysis. In addition, it could be used as a potential treatment for bladder incontinence, a condition that currently affects over 3.3 million Canadians (CCF, 2010). Chondroitin sulphate has been shown to restore bladder function by restoring the bladder permeability barrier (Hauser et al., 2009), and elastin is a component of the connective tissue fibres in the bladder wall (Cortivo, 1981). Therefore, a material combining these two components would provide a novel biomaterial that could potentially restore bladder function and potential repair bladder tissue.

2.9 Bacterial Expression

Bacterial expression systems are a common method of production of recombinant proteins. This method of protein production is highly attractive as it is an inexpensive and readily available system, most bacteria are able grow rapidly and to high densities, and their genetics are well characterized (Terpe, 2006). The main procedure used for the *in vitro* production of elastin-like polypeptides is by recombinant techniques involving the growth of transformed *E. coli* bacterial cells in a fermentor.

E. coli is the most commonly used bacteria for recombinant protein production. It is a very well known and established expression system and can be used for large-scale production (Terpe, 2006). One of the only disadvantages of using *E. coli* to produce recombinant proteins for use in tissue engineering is the potential accumulation of lipopolysaccharides (LPS), also known as endotoxins (Petsch & Anspach, 1999). While LPS is potentially problematic, proteins

that have been contaminated with it can be further purified to become endotoxin-free. Recombinant proteins that are overexpressed by *E. coli* collect in the cell cytoplasm, which has shown to produce high yields. Typical *E. coli* expression systems use the BL21 strain, a protease-deficient strain that has shown to be a good host for the expression of recombinant proteins. (Terpe, 2006).

In DNA cloning, recombinant DNA molecules are formed *in vitro* by inserting DNA fragments into vector DNA molecules that can replicate within a host cell (Lodish et al., 2003). One of the most common types of vectors used in *E. coli* host cells is plasmids, which are circular, double-stranded DNA molecules that are separate from a cell's chromosomal DNA. These plasmids have been optimized for their use as vectors such that they are engineered to contain three regions essential for DNA cloning: a replication origin; a selection marker, typically a drug-resistant gene; and a region in which external DNA fragments can be inserted (Lodish et al., 2003). Any DNA sequence inserted into a plasmid vector is replicated along with the rest of the plasmid DNA.

When the vectors are mixed with *E. coli* cells, only a small fraction of the cells will actually take up the plasmid DNA, a process known as transformation (Lodish et al., 2003). Selection of the cells that actual take up the plasmid and are able to express the DNA fragment is relatively easy through the use of the selection marker in the vector. For example, the pGEX vector that has been used to produce ELPs in the Woodhouse laboratory contains the ampicillin-resistance gene amp^r, such that when the cells are grown in an ampicillin-containing medium, only those transformed by the plasmid will survive.

The mass production of recombinant proteins using a bacterial expression system is commonly carried out through fermentation. A bioreactor is used as the vessel in which *E. coli*

cells can be reproduced under optimal growth conditions. Bacteria such as *E. coli* undergo binary fission to replicate and typically experience exponential growth (Zwietering et al., 1990). There are four main phases of exponential growth: the lag phase where the bacteria adapt to the growth conditions, the exponential phase that consists of cell doubling, the stationary phase where cell growth plateaus, and finally the death phase, where the bacteria run out of nutrients and die (Zwietering et al., 1990). During fermentation, conditions are monitored to ensure maximal growth and the process is completed before the final death phase begins. Growth of the cells is analyzed by monitoring the optical density of the fermentation media, which can be used to create a bacterial growth curve. The creation of the cell growth curve is important for protein expression as it gives an indication of cell density, which directly relates to protein yield.

Production of ELPs in the Woodhouse Lab has been accomplished using an *E. coli* BL21 bacterial expression system (Bellingham et al., 2001; Keeley et al., 2002; Bellingham et al., 2004; Woodhouse et al., 2004). Based on this previous work as well as the advantages of using bacterial expression described above, a bacterial expression system using *E. coli* BL21 containing pGEX vectors will be used in this study to produce the ELP1-CSBDs.

Chapter 3

Materials and Methods

3.1 Materials

All materials were purchased from Sigma-Aldrich Canada (Oakville, ON) unless otherwise stated.

3.2 ELP-CSBD Constructs

The ELP1 polypeptide has previously been expressed, produced, and purified by the Woodhouse Lab (Bellingham et al., 2001). The ELP1-CSBD1 construct is a modified version of the ELP1 polypeptide. ELP1 corresponds to exons 20-21-23-24 of the human elastin gene. The construct was produced by sequentially changing the crosslinking domains coded by exons 21 and 23 and replacing them with the CSBD1 peptide (IKFIHRKYRKHG) coding sequence. The ELP1-CSBD2 construct was produced in a similar manner but using the CSBD2 peptide sequence (RVFKRYKRWLHG). These constructs are all designed as glutathione S-transferase (GST) fusion proteins.

3.3 Transformation of E. coli with Recombinant ELP1-CSBD1 Vector

Previous work completed in the Woodhouse Lab includes the successful modification of the ELP1 DNA sequences to include the CS binding sequences, followed by the insertion of these ELP1-CSBD1 and ELP1-CSBD2 sequences into pGEX-2T expression vectors. The procedure used to transform the pGEX-2T vectors into *E. coli* BL21 cells, completed by Ji (2009) and Parrag (2010), is outlined as follows. The modified ELP construct was first subcloned into the pGEX-2T expression vector (GE Healthcare Biosciences Inc., Baie d'Urfé, QC). This purified recombinant pGEX-2T vector was then transformed into T7 Express lysY/I^q competent *E. coli*

BL21 cells (New England Biolabs Inc., Pickering, ON). The competent cells were thawed on ice for 20 minutes and were transferred to a transformation tube. Approximately 50-100ng of the purified plasmid DNA was added to the cells and the tubes were placed on ice for 30 minutes. The cells were subsequently heat-shocked at 42°C for 5 minutes and room temperature SOC Outgrowth Medium (New England Biolabs Inc.) was added to the tubes. The cells were incubated at 37°C with vigorous shaking for 1 hour, streaked onto LB-agar selection plates containing 100 µg/mL ampicillin sodium salt (IBI Scientific, Peosta, IA), and then were incubated overnight at 37°C. Individual colonies were selected, inoculated into 5mL of LB medium containing ampicillin (100 µg/mL), and grown overnight in a shaker incubator at 37°C and 250 rpm. The following day, 4mL of the overnight culture was used to isolate DNA as described below, while the remaining culture was used to make glycerol cell stocks. Sterile, autoclaved glycerol was added to the cell solution in a 1:5 ratio and cells were frozen and stored at -80°C until use.

Successful transformation of the recombinant vector and confirmation of the desired DNA insert sequence was verified by gel electrophoresis and DNA sequencing. The overnight cultures of the transformed *E. coli* were spun down at 6,800 x g for 20 min at 25°C and DNA was isolated using a QIAPrep Spin Miniprep Kit (QIAGEN Inc., Mississauga, ON) according to manufacturer's instructions. The isolated DNA, in concentrations ranging from 30-80 ng/µl, was sequenced by ACGT Corporation (Toronto, ON). For DNA gel electrophoresis, the restriction enzymes *Bam*HI and *Eco*RI (Invitrogen, Carlsbad, CA) were used to digest the ELP1-CSBD1 and -CSBD2 inserts from the pGEX-2T vector. The restriction enzymes were added at a final concentration of 0.5 U/µl for both enzymes to a solution containing 60-120ng of isolated DNA, React 3 buffer (Invitrogen), and deionized water. The solutions were incubated at 37°C for 1

hour and were subsequently run on a 2% agarose gel at 125V for 1 hour in TAE buffer (Invitrogen). The gel was stained using SYBR® Gold nucleic acid gel stain (Invitrogen).

3.4 Expression and Purification of the ELPs

Small ice crystals of the transformed *E. coli* glycerol stock were added to 250mL shaker flasks with 100mL of LB medium containing ampicillin (100µg/mL). The cells were incubated overnight at 37°C and 200 rpm. A 10 L Bioflo® 110 bioreactor (New Brunswick Scientific, Edison, NJ) was prepared with 10 L of 2 x YT broth containing 100µg/mL ampicillin and 20g/L glucose. The system was inoculated with the overnight shaker flask culture and cell expansion proceeded at 37°C, 35% dissolved oxygen, 200-500 rpm agitation, and a pH of 7.0. Using an Evolution 300 UV-Vis Spectrophotometer (Fisher Scientific, Ottawa, ON), cell growth was monitored by measuring the optical density at 600 nm (OD_{600}), the standard wavelength used to observe the density of cells in bacterial culture. When the OD_{600} became greater than 5 (after approximately 5 hours), isopropyl-K-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5mM was added to induce expression of the polypeptides. After an additional 2-2.5 hour incubation period, the cells were harvested from the vessel, centrifuged at 6,800 x g for 20 minutes at room temperature, and the cell pellet was frozen at -20°C until further processing.

3.4.1 Purification of ELP1-CSBDs

The same purification procedure was used for each ELP1-CSBD1, ELP1-CSBD2, and ELP1 unless otherwise stated. Cell lysis and cleavage of the GST-tag from the ELP1-CSBD was carried out using cyanogen bromide (CNBr) at 50mg/mL in nitrogenated 70% formic acid overnight. The resulting solution was then dialyzed using 3,500 molecular weight cut-off (MWCO) snakeskin dialysis tubing (Fisher Scientific) against deionized water and then in 20mM sodium acetate (NaOAc) buffer at pH 5. The subsequent mixture was centrifuged using an

Avanti® J-26 XPI High-Performance Centrifuge (Beckman Coulter Canada, Mississauga, ON) at 15,000 x g for 40 minutes at 4°C. The remaining cell pellet was discarded and the supernatant was collected and filtered using Whatman® 1 filter papers. The clarified cell extract was stored at 4°C until further purification.

The cell extract solution was purified by ion exchange chromatography using SP Sepharose Fast Flow resin (GE Healthcare Biosciences Inc.). The cell extract was loaded onto the chromatography column at a volume based on the size of the column and the binding capacity of the resin (50mg protein/mL resin in buffer). Solutions containing different concentrations of NaCl were used in a stepwise series in order to purify and elute the protein. Table 1 shows the different concentrations of NaCl used to elute ELP1, ELP1-CSBD1, and ELP1-CSBD2. An Evolution 300 UV-Vis Spectrophotometer was used to evaluate protein concentration and relative purity of the samples off the column by measuring the optical density of the fractions at both 230nm and 280nm, corresponding to UV absorption of peptide bonds and aromatic amino acids, respectively. Fractions were retained if the ratio of 230nm/280nm was 7 or above.

Table 1: Concentrations of sodium chloride (NaCl) solutions used during ion exchange chromatography. Different concentrations were used to eliminate unwanted contaminants, elute ELP1, ELP1-CSBD1, and ELP1-CSBD2 polypeptides respectively, and finally to clean and strip the SP Sepharose column.

[NaCl]\ELP	ELP1	ELP1-CSBD1	ELP1-CSBD2
Elution of Contaminants	20 mM NaCl	80 mM NaCl 200 mM NaCl 250 mM NaCl	80 mM NaCl 300 mM NaCl
ELP Elution	80 mM NaCl	300 mM NaCl	600 mM NaCl
Column stripping	1500 mM NaCl	1500 mM NaCl	1500 mM NaCl

The collected fractions containing the eluted ELP were dialyzed in 50mM acetic acid in deionized water and analyzed through mass spectroscopy and amino acid analysis in order to assess expression and purity. Mass spectroscopy was carried out at the Protein Function Discovery facility at Queen's University (Kingston, ON) using a SCIEX Voyager DE Pro MALDI -TOF Mass Spectrometer operating in linear mode and at the Advanced Protein Technology Centre (APTC) at the Hospital for Sick Children (Toronto, ON) using an Applied Biosystems/MDS Sciex API QSTAR XL Pulsar MALDI QTOF Mass Spectrometer. Amino acid analysis was also conducted at the APTC, using the Waters Pico-Tag System. The purified proteins were stored in 50mM acetic acid in the -20°C freezer, and were lyophilized using a Labconco Freezone® Freeze Dry System as needed.

3.5 Coacervation

Coacervation studies were carried out on the ELP1-CSBD1 polypeptide using a Thermo Evolution 300 spectrophotometer equipped with a temperature controller (Fisher Scientific). The DNA Method in the Vision Pro Thermo Electron UV-Vis Spectrophotometer Software (Fisher Scientific) was used to perform sample analysis during temperature ramping. ELP1-CSBD1 was dissolved in coacervation buffer (50 mM Tris, 1.5 M NaCl, 1 mM $CaCl_2$) at pH 9.5 and at concentrations ranging from 2 - 100μM and were stored at 4°C for at least 20 minutes to ensure the peptide was fully dissolved. Studies were conducted based on methods created by Bellingham (2001). Solutions were placed in a quartz cuvette (Hellma (Canada) Limited, Concord, ON) and were inserted into the sample cell of the temperature controller and allowed to equilibrate at 15°C for 5 minutes. The temperature was then ramped at a rate of 1°C per minute with gentle stirring. The absorbance at 440 nm was monitored every minute for at least 50 minutes until it appeared that coacervation was complete. Coacervation was identified as the

onset of turbidity appearing as an increase in absorbance. The coacervation temperature (T_c) was taken as the temperature corresponding to the intersection of the tangent to the curve before coacervation with the tangent to the initial increase in absorbance on a temperature vs. absorbance plot (Figure 5). The slope of the steepest part of the coacervation curve was used to determine the rate of coacervation. Coacervation experiments used polypeptides produced from separate batches of expressed and purified protein in order to take into account variability between batches.

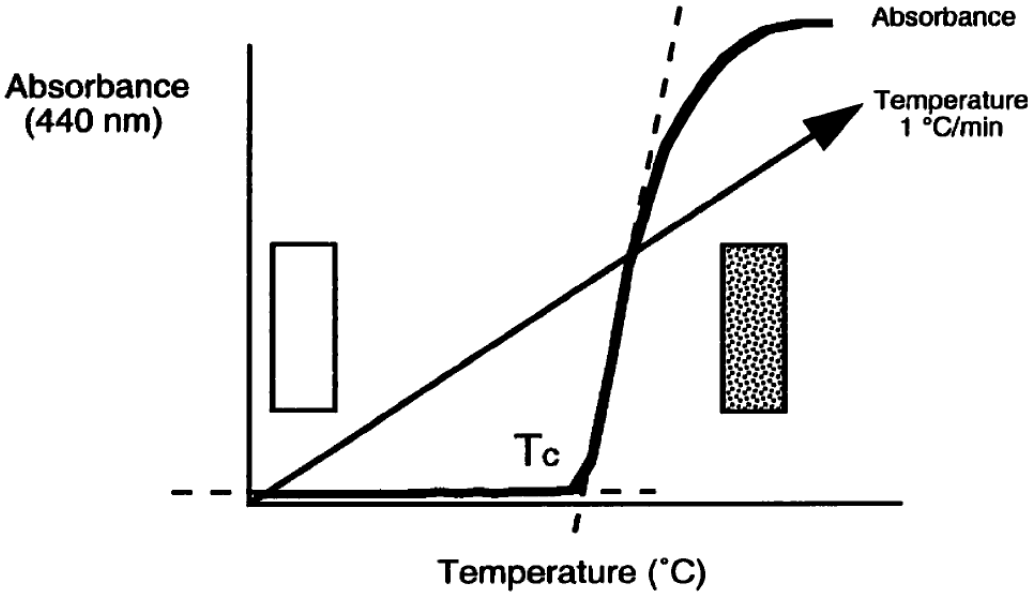


Figure 5: The process of coacervation, studied by monitoring turbidity by light scattering at 440nm over a range of temperatures. Before coacervation, the protein solution is clear. During coacervation, the solution becomes cloudy, resulting in an increase in absorbance. The temperature is increased at a rate of 1°C per minute. The coacervation temperature, T_c, is defined as the onset of turbidity and is determined as the temperature corresponding to the intersection of the tangent to the curve prior to coacervation and the tangent to the initial increase in absorbance (dashed lines). Adapted from Bellingham (2001).

Coacervation studies using ELP1-CSBD1 were compared to those conducted by Bellingham using ELP1 (2001). In those studies, polypeptides were dissolved at analogous concentrations in coacervation buffer (50 mM Tris, 1.5 M NaCl, 1 mM $CaCl_2$) but instead at pH

7.5. A Cary 3 spectrophotometer equipped with a temperature controller was used to monitor coacervation (Varian Inc., Victoria, Australia). Similar to these studies, the temperature was ramped at a rate of 1°C per minute with gentle stirring and the absorbance at 440nm was measured every minute. Coacervation temperature and rate were measured in the same manner as outlined above.

To study the effect of chondroitin sulphate on coacervation, ELP1-CSBD1 and chondroitin-6-sulphate (C6S) (MW ~20000 g/mol) from shark cartilage were separately dissolved in coacervation buffer (50 mM Tris, 1.5 M NaCl, 1 mM $CaCl_2$, pH 9.5) at concentrations of 200, 100, and 50 μM. Prior to performing coacervation, equal amounts of ELP1-CSBD1 and CS were added to the quartz cuvette resulting in final concentrations of 100, 50, and 25 μM of each, ensuring a 1:1 molar ratio was maintained. Coacervation was then performed as previously described. As a control, the effect of CS on the coacervation of ELP1 was also studied and experiments were carried out as above. Experiments studying the effect of heparin on coacervation were also conducted. Using heparin sodium salt from porcine intestinal mucosa (MW ~18000 g/mol), the same procedure as with the chondroitin sulphate studies was performed.

A total of six repeats were conducted for each condition studied unless otherwise stated. All statistics were performed using StatPlus LE (Version 2009) (AnalystSoft Inc., Alexandria, VA). Linear regression analysis was performed on coacervation data to determine coacervation temperature and rate. Standard variation for the coacervation data was determined and differences between coacervation trials were assessed by analysis of variance (ANOVA) where p-values lower than 0.05 were considered significant. Statistical data was obtained with n=6 unless otherwise stated.

Chapter 4

Results and Discussion

4.1 Method Development

The ELP1-CSBD1 and ELP1-CSBD2 peptides have not been produced before and therefore it was necessary to develop and confirm the production methodologies. These protocols were based on the original elastin polypeptide procedures (Bellingham et al., 2001) and refined for the ELP1-CSBDs, which have different properties than basic ELP1 (Table 2).

4.2 ELP-CSBD Expression Constructs

The elastin-like polypeptide CSBD constructs were designed as glutathione S-transferase (GST) fusion proteins. This is based on the original constructs designed for ELP1 that contained the GST tag for purification reasons. While the CSBD constructs contained the GST tag, it was cleaved off during the early stages of purification as ion exchange chromatography has shown acceptable results in the purification of the ELPs and is a less expensive method than one involving the affinity chromatography associated with the GST tag.

Table 2 shows a summary of the theoretical characteristics of each polypeptide including their molecular weight (MW), isoelectric point (pI), and hydropathy value. The theoretical molecular weights and isoelectric points were determined based on the expected amino acid sequences of the ELPs using the Basic Local Alignment Search Tool (BLAST), while the mean hydropathies were calculated using the grand average of hydropathy (GRAVY) value for protein sequences. The GRAVY value was defined by the sum of hydropathy values of all the amino acids in each of the ELP1-CSBD sequences divided by the length of the protein (Kyte and Doolittle, 1982).

The molecular weight of ELP1 is slightly higher than that of ELP1-CSBD1 and ELP1-CSBD2 because the crosslinking sequence in ELP1 is much larger than the binding sequences in the ELP1-CSBDs. The pI values for both ELP1-CSBD1 and ELP1-CSBD2 were higher than that of ELP1. The hydropathy values reflect the overall hydrophobicity of the polypeptides, which were generally the same for all ELP Types. This is reasonable as the crosslinking domains vary between the peptides, while the hydrophobic domains are consistent between them.

Table 2: Theoretical properties of ELP1, ELP1-CSBD1, and ELP1-CSBD2. Theoretical properties were determined from the amino acid sequences of the ELPs using [1] the National Center for Biotechnology Information BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or [2] the grand average of hydropathy (GRAVY) calculator (http://www.gravy-calculator.de).

ELP Type	Molecular Weight (Da)[1]	pI[1]	Hydropathy[2]
ELP1	9848	9.84	0.91
ELP1-CSBD1	8457	11.17	0.84
ELP1-CSBD2	8520	11.73	0.85

4.3 Transformation of E. coli with the Recombinant ELP1-CSBD Vectors

Recombinant pGEX-2T expression vectors carrying the ELP1-CSBD inserts were successfully transformed into *E. coli* BL21 cells for expression of the recombinant polypeptides. The cells were plated on ampicillin-containing plates to select for cells that were successfully transformed. The pGEX-2T vector contains an ampicillin resistant gene that allows for selection of cells that have taken up the vector. Cell colonies were formed in all sample groups while no cell growth was observed for the negative control, consisting of cells undergoing the same transformation procedure but in the absence of the pGEX-2T vector. Individual colonies were expanded overnight with the OD_{600} reaching maximum values from 1.3 to 1.7 and DNA was isolated from the cultures, with concentrations ranging from approximately 30 to 80 ng/µl.

To determine the success of transformation, gel electrophoresis and DNA sequencing were used to analyze the isolated DNA. Figure 6 shows the results of gel electrophoresis from the isolated DNA cut with the restriction enzymes *Eco*RI and *Bam*HI. In all samples tested, two bands were identified; a DNA segment greater than 3000 bp and a short segment approximately 300 bp long. These DNA fragments correspond well to the size of the pGEX-2T vector (~4900 bp) and the ELP1-CSBD inserts (291 bp, including a methionine residue at the start of the sequence), suggesting successful transformation of the recombinant expression vectors. Results from DNA sequencing further support this finding and confirm the transformed cells contain the ELP1-CSBD inserts with the correct sequence. BLAST-Align analysis results corresponding to the theoretical ELP1-CSBD DNA sequences and the DNA sequencing results on the isolated DNA show a 100% base pair match for each of the ELP1-CSBD sequences (Appendix A).

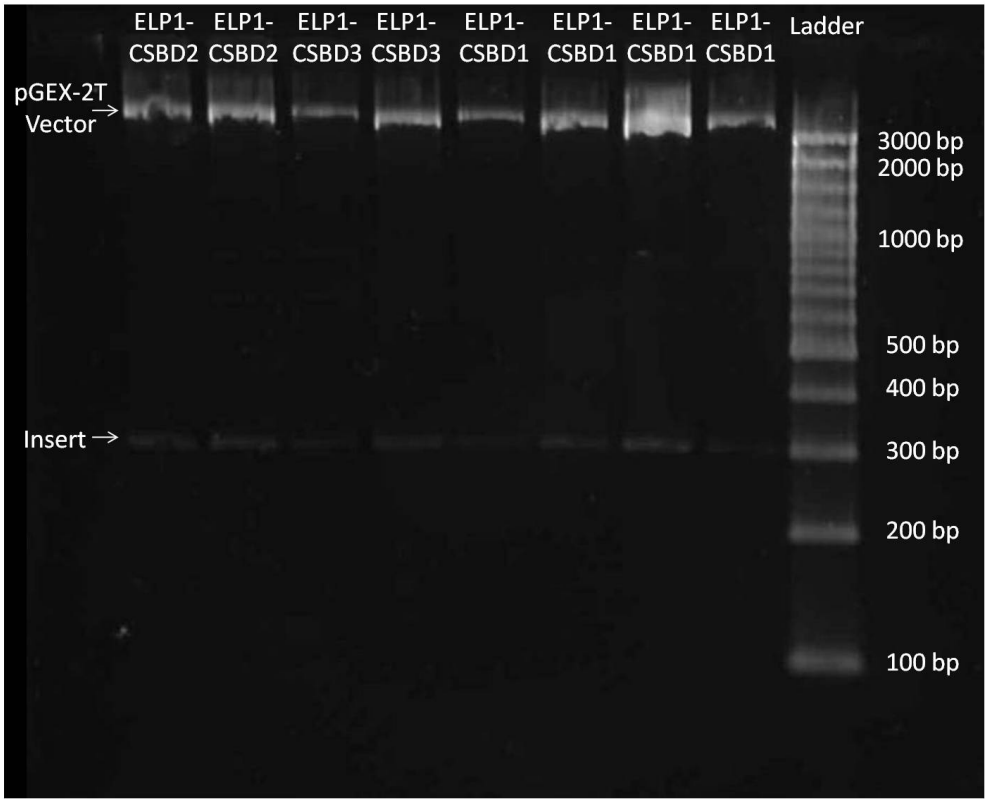


Figure 6: DNA gel electrophoresis of the isolated DNA from transformed *E. coli* following a restriction digest with *Bam*HI and *Eco*RI.

4.4 Expression and Purification of ELP1-CSBD1

All constructs were expressed using an *E. coli* BL21 bacterial system. The growth of the recombinant *E. coli* BL21 cells containing the ELP1-CSBD1 and ELP1-CSBD2 expression vectors were investigated. Figure 7 shows a typical cell growth curve for the expression of each ELP1-CSBD1 and ELP1-CSBD2. The cells entered the exponential phase approximately 3 hours after inoculation, and were typically in the middle of exponential growth after about 5 hours in total. Induction of ELP expression was initiated by IPTG at this time. The fermentation was subsequently stopped 2-2.5 hours after induction when it was found that the cells entered their stationary phase and cell growth plateaued. These times for induction and termination were

relatively consistent across the four fermentation batches of each CSBD polypeptide with induction occurring after 5 hours of growth and completion of the fermentation after 7-8 hours.

The results of the fermentations conducted for ELP1-CSBD1 and ELP1-CSBD2 are shown in Appendix B. The cell pellet yield based on four fermentation runs for the expression of ELP1-CSBD1 ranged from 162 to 212g with an average yield of 172g. The maximum absorbance reached was an average of 10.8. The average yield of four fermentations of ELP1-CSBD2 was 180g with a range of 138g to 211g, while the average maximum absorbance was 10.2. It is apparent from these results that the expression of ELP1-CSBD1 and ELP1-CSBD2 was very similar.

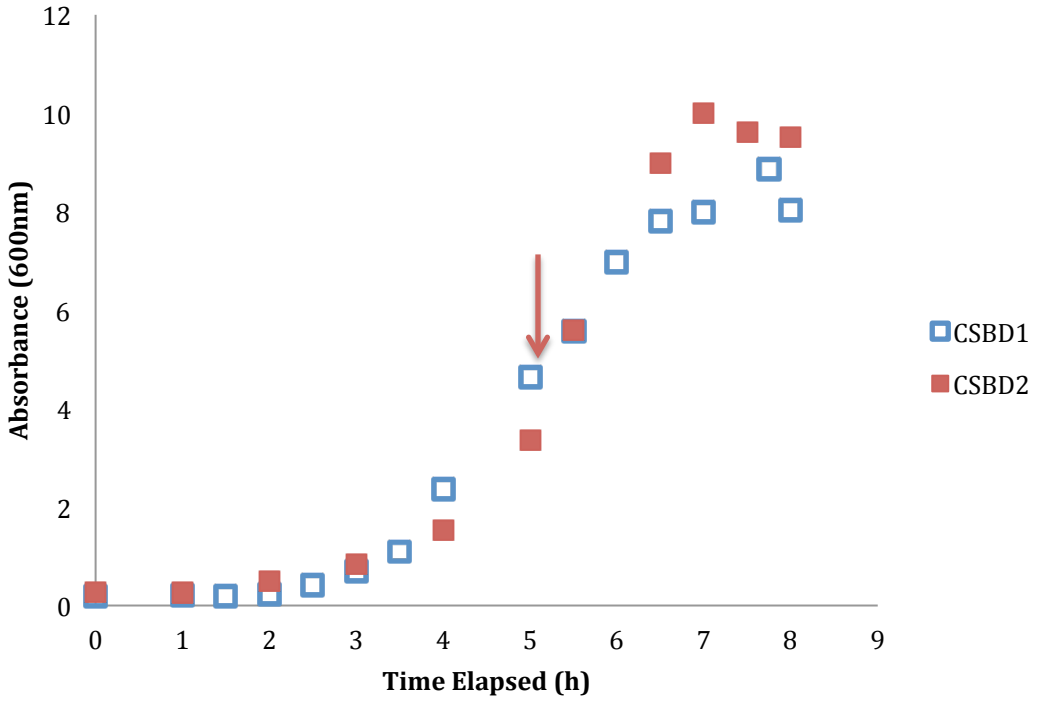


Figure 7: Typical cell growth curves of *E. coli* BL21 transformed with recombinant ELP1-CSBD1 and ELP1-CSBD2 plasmid vectors. The arrow indicates the average induction time where IPTG was added.

Following fermentation, the ELP1-CSBDs were processed using CNBr and 70% formic acid to lyse the cells and cleave the GST tag from the recombinant polypeptide. CNBr

hydrolyzes peptide bonds at the C-terminus of methionine residues, and was used as there is only one methionine residue found at the beginning of both ELP1-CSBD sequences. The ELP1-CSBDs were subsequently purified through ion exchange chromatography using the strong cation exchange resin SP Sepharose ($-O-CH_2CHOHCH_2OCH_2CH_2CH_2SO_3^-$). Initial purification of the ELP1-CSBDs was tested using a small column containing approximately 50 mL of resin. In order to determine purification protocols for the ELP1-CSBDs, the polypeptides were eluted from the column at different NaCl concentrations in sodium acetate buffer using a direct correlation between their theoretical isoelectric points (pI) and elution NaCl concentrations. Once a new protocol was established for each peptide, this was scaled up to a column containing approximately 300 mL of resin. The protocols for ELP1-CSBD1 and ELP1-CSBD2 have been confirmed on a large scale, and are outlined below.

The unpurified ELP1-CSBDs were dialyzed in sodium acetate buffer at pH 5, significantly below the theoretical pI of the polypeptides (Table 2) but within the functional operating range of the resin, to ensure that the polypeptides were positively charged for ion exchange. The ELPs were loaded onto the columns and were eluted off at varying NaCl concentrations. In each case, wash steps using NaCl concentrations below the elution concentration were used to remove undesirable contaminants. A series of different wash steps were tested for each polypeptide in order to obtain the highest purity. As expected, a direct relationship was observed between theoretical pI and the NaCl concentration used to elute ELP1 and the ELP1-CSBDs. Final NaCl concentrations used for the purification of each polypeptide and the corresponding purities are shown in Table 3. ELP1, which has a theoretical pI of 9.84, was eluted at an 80 mM NaCl concentration, while ELP1-CSBD1, which has a high theoretical pI

of 11.17, was eluted at a 300 mM NaCl concentration and ELP1-CSBD2 (pI 11.73) was eluted at 600mM NaCl.

Table 3: Experimental Properties of ELP1, ELP1-CSBD1, and ELP1-CSBD2. The maximum purity obtained in this research for each ELP type is shown. Purity of the ELPs was determined by comparing theoretical amino acid composition of each ELP to that determined experimentally by amino acid analysis.

ELP Type	NaCl Wash Concentrations	NaCl Elution Concentration	Maximum Purity Obtained (%)
ELP1	20 mM	80 mM	97.8
ELP1-CSBD1	80 mM 200 mM 250 mM	300 mM	94.4
ELP1-CSBD2	80 mM 300 mM	600 mM	56.3

Successful expression and purification of the ELP1-CSBDs were confirmed by mass spectroscopy and amino acid analysis. The molecular mass of the polypeptides was determined by mass spectrometric analysis, and the results agree with expected values. For the ELP1-CSBD1 peptide (Figure 8), the spectrum showed two dominant peaks, one at a mass to charge (m/z) ratio of 8457.9 and the other at 4229.09. These values correspond to the theoretical mono-protonated (8458) and di-protonated (4229.5) forms of ELP1-CSBD1 respectively, thus confirming the identity of the ELP1-CSBD1 polypeptide. Relatively few other peaks were observed suggesting successful expression and purification of the ELP1-CSBD1. For the ELP1-CSBD2 peptide (Figure 9), the spectrum showed one dominant peak, this time at a mass to charge ratio of 8520.3. There was no di-protonated peak in this spectrum simply because of differences in the method of analysis, as ELP1-CSBD2 was analyzed using a QSTAR MALDI QTOF spectrophotometer that detects singly charged ions while the ELP1-CSBD1 spectra had been

analyzed using a basic MALDI -TOF spectrophotometer that can detect up to three charges on a protein. This difference in spectrophotometers used was simply due to equipment availability. While it is apparent based on the spectrum in Figure 9 that the ELP1-CSBD2 was in fact eluted at 600mM NaCl, there were still extra peaks in the spectrum that are likely corresponding to contaminating molecules, implicating that the purification was not optimized for this protein.

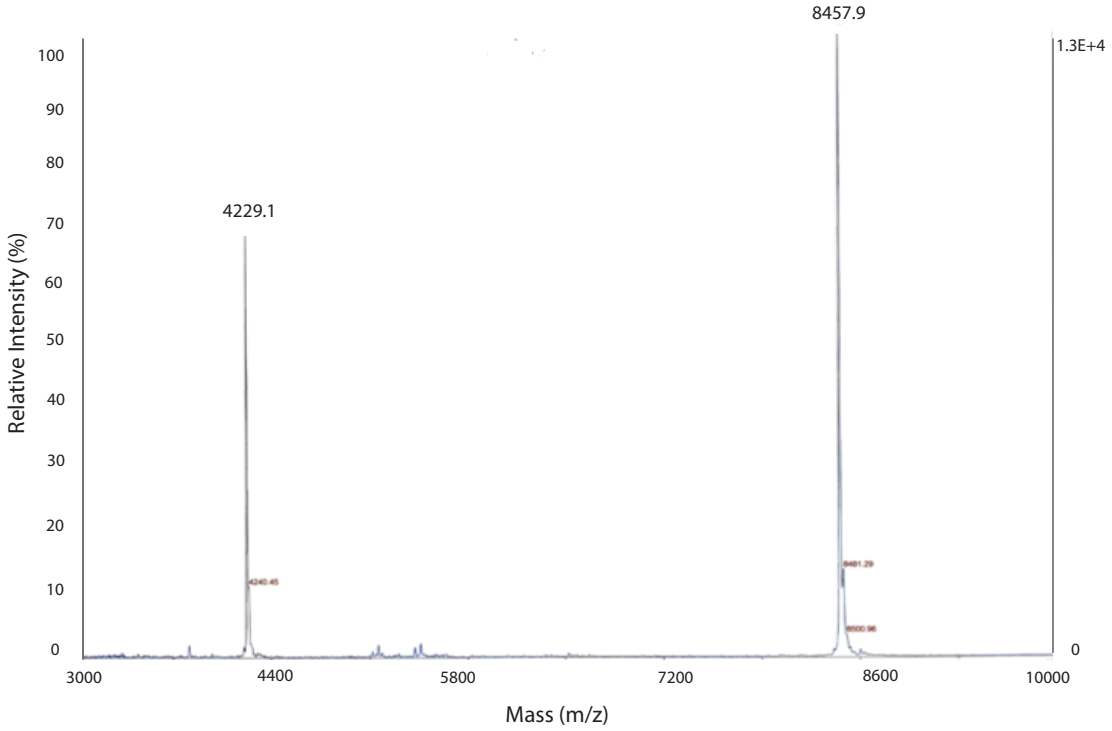


Figure 8: Representative MALDI-TOF mass spectra of purified ELP1-CSBD1. Analysis was performed using a SCIEX Voyager DE Pro MALDI -TOF Mass Spectrometer. Two prominent peaks are identified in the spectrum at a mass of 4229 and 8458 that correspond to the mono- and di-protonated forms of the ELP, respectively. Relatively few other peaks are observed suggesting successful purification.

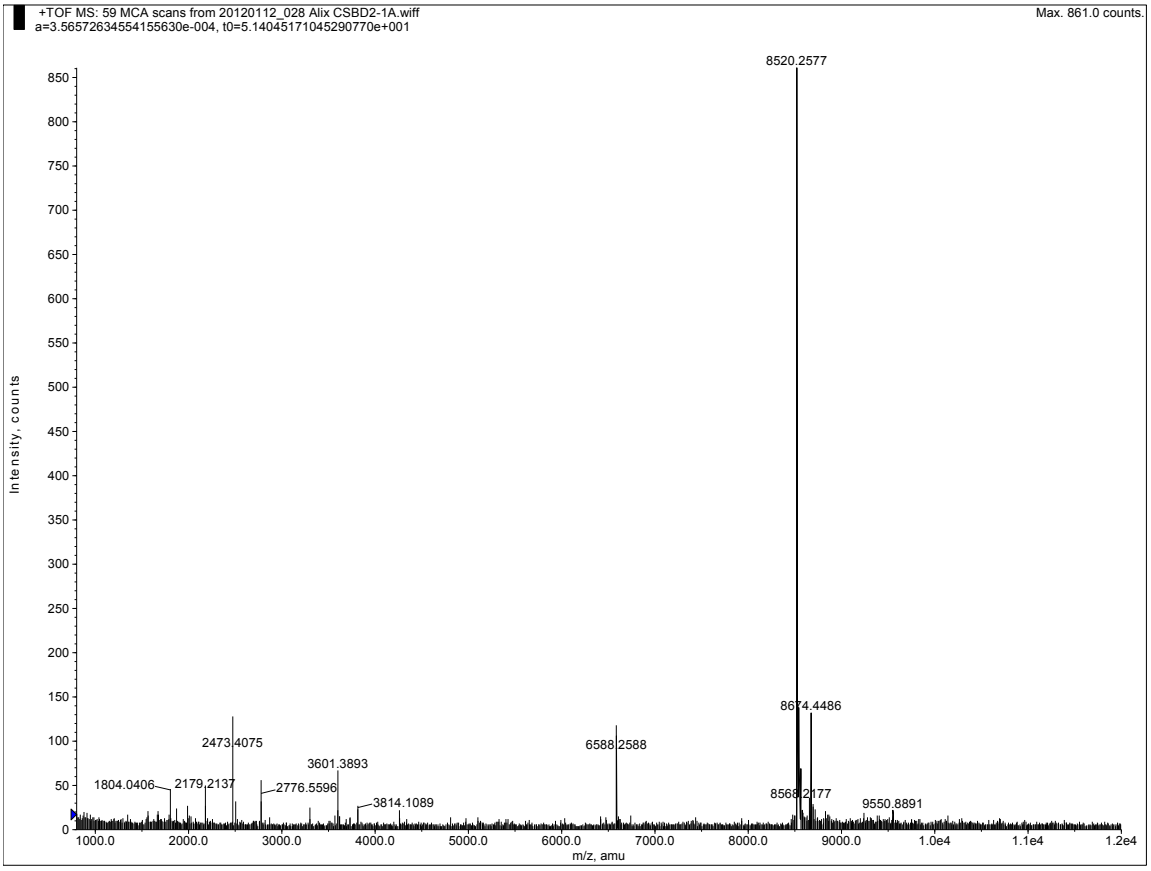


Figure 9: Representative MALDI-TOF mass spectra of purified ELP1-CSBD2. Analysis was performed using an API QSTAR XL Pulsar MALDI QTOF Mass Spectrometer. One prominent peak is identified in the spectrum at 8520 that corresponds to the mono-protonated form of the ELP. It is important to note that even after purification, there are still a few other peaks observed, corresponding to contaminating molecules.

The purities of the isolated polypeptides were assessed through amino acid analysis by comparing expected amino acid compositions to actual compositions. Using a spreadsheet created in the Keeley laboratory (Appendix B), the purity of the protein samples (Table 3) were determined by comparing calculated molar ratios between the amino acid residues to the expected molar ratios (Table 4, Table 5). This analysis identified an average purity of 84.8% from a total of 24 purification attempts of ELP1-CSBD1, with a maximum purity identified to be as high as 94.4%. Acceptable amino acid results have a purity of 85% and higher, thus confirming that the purification protocols are successful for ELP1-CSBD1. In regards to ELP1-CSBD2, the analysis

identified a maximum purity of only 56.3% from a total of 9 different purification attempts. This is not deemed an acceptable result, so therefore it can be speculated that while the expression of ELP1-CSBD2 did in fact work, the purification protocols will need to be investigated further.

A reason for the low purity of ELP1-CSBD2 could be not including enough NaCl wash steps in the ion exchange column purification procedure; however, separate trials were also conducted where solutions of 400mM NaCl or 500mM NaCl were added to the purification protocol, but the ELP1-CSBD2 protein was merely found to elute at these steps and there were many more contaminants present in the flow through. Therefore, the wash steps in the protocol proposed for ELP1-CSBD2 (Table 3) are the most promising at this point, although further optimization is necessary in order to achieve a higher purity. Based on the low purities achieved, ELP1-CSBD2 was not investigated past this point.

Table 4: Expected amino acid contents for ELP1 and the ELP1-CSBDs.

Amino Acid	*ELP1*	*ELP1-CSBD1*	*ELP1-CSBD2*
Ala (A)	26	8	8
Arg (R)	0	2	3
Gln (Q)	2	0	0
Glu (E)	1	0	0
Gly (G)	34	32	32
His (H)	0	2	1
Ile (I)	3	5	3
Leu (L)	2	2	3
Lys (K)	4	3	2
Phe (F)	2	2	2
Pro (P)	16	15	15
Ser (S)	1	1	1
Thr (T)	1	0	0
Trp (W)	0	0	1
Tyr (Y)	1	1	1
Val (V)	24	23	24
SUM	**117**	**96**	**96**
Negatively charged residues (Glu, Asp)	1	0	0
Positively charged residues (Lys, Arg, His)	4	7	6

Table 5: Representative observed amino acid content for ELP1-CSBD1 compared to the expected content based on molar ratios determined through amino acid analysis (Appendix B).

Amino Acid	*Expected Molar Ratio*	*Observed Molar Ratio*
Ala (A)	8	8.2
Arg (R)	2	2.8
Asp (D)	0	0.6
Gln (Q) + Glu (E)	0	0.8
Gly (G)	32	31.9
His (H)	2	2.4
Ile (I)	5	4.5
Leu (L)	2	2.2
Lys (K)	3	2.7
Phe (F)	2	2.0
Pro (P)	15	15.0
Ser (S)	1	1.0
Thr (T)	0	0.3
Trp (W)	0	0
Tyr (Y)	1	1.1
Val (V)	23	20.3
SUM	**96**	**96**
Purity	100%	94.4%
Negatively charged residues (Glu, Asp)	0	0
Positively charged residues (Lys, Arg)	5	5

Approximately 10mg of ELP1-CSBD1 could be recovered on average through lyophilization of 45mL of the processed and purified protein solution, corresponding to ~2.5% of cell pellet from the fermentation. Therefore, it is anticipated that a yield of approximately 400 mg of purified ELP1-CSBD1 may be produced from the 10 L fermentation that had a cell pellet of approximately 170g. This implies that the average yield is approximately 2.4mg ELP1-CSBD1/g of cell pellet, or 40mg ELP1-CSBD1/L of culture. This is better than the yields

determined for ELP1, which Bellingham et al. (2001) found to range from 2-10mg of polypeptide per litre of culture. Reasons for this increase in the yield of the purified protein likely include the use of a larger, more automated and controlled bioreactor system, and differences in purification techniques, as Bellingham used affinity chromatography to purify ELP1 while ion exchange chromatography was used for ELP1-CSBD1. In addition, differences in codon bias, or the frequency of occurrence of certain codons in the coding DNA, between the protein genes and *E. coli host* can limit expression (Makoff et al., 1989; Kurland, 1991). Stephen et al. (1995) have specifically demonstrated that matching codon bias between a synthetic tropoelastin and its host organism can support high expression of recombinant sequences. Therefore, because of the differences in amino acid sequence between ELP1 and ELP1-CSBD1, there could be a better fit of codon bias between ELP1-CSBD1 and *E. coli* leading to a higher level of expression of this protein.

Overall, ELP1-CSBD1 was successfully expressed using *E. coli* and purified using the biochemical techniques described in the Methods section. While ELP1-CSBD2 was successfully produced using the same expression system, it was not purified to as high of purity as ELP1-CSBD1.

4.5 Coacervation

Coacervation has commonly been used as a basic measure of the propensity for elastin to undergo aggregation. Bellingham et al. (2001) have previously characterized the ability of the ELP1 peptide to self-aggregate *in vitro* under a variety of conditions. In order to determine if the ELP1-CSBD1 peptide behaved in a similar fashion to ELP1, studies were conducted to determine the ability of the new polypeptide to aggregate using coacervation as a measure of the self-assembly process.

Initial coacervation studies were carried out in standard coacervation buffer (50mM Tris, 1.5M NaCl, 1mM $CaCl_2$) at pH 9.5 and varying polypeptide concentrations (adjusted for purity using the results from the amino acid analysis). All concentrations of ELP1-CSBD1 studied (with the exception of 2.0μM) were able to undergo coacervation suggesting that ELP1-CSBD1 is able to self-assemble. The generated coacervation curves for each polypeptide concentration are shown in Figure 10. Quantitative results for each concentration are summarized in Table 6.

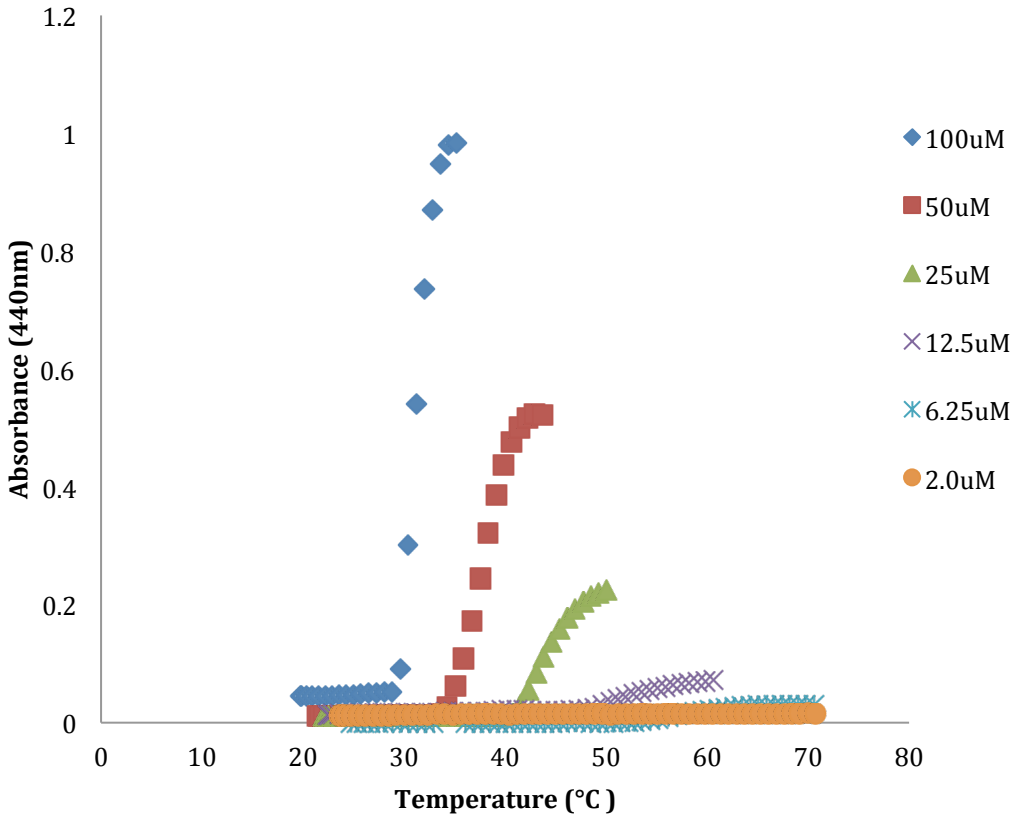


Figure 10: Effect of polypeptide concentration on coacervation temperature of ELP1-CSBD1. Coacervation studies were carried out in standard coacervation buffer (50mM Tris, 1.5M NaCl, 1mM $CaCl_2$) at pH 9.5. Average coacervation curves are shown for each concentration. Concentrations were varied as indicated, with all other conditions identical. A minimum of 6 coacervation trials was conducted for each polypeptide concentration.

Table 6: Coacervation temperatures, rates of coacervation and maximum absorbance values corresponding to the different ELP1-CSBD1 concentrations studied. All values are stated as mean ± standard deviation. Coacervation studies were carried out in standard coacervation buffer (50mM Tris, 1.5M NaCl, 1mM $CaCl_2$) at pH 9.5. A minimum of 6 coacervation trials was conducted for each concentration. ELP1-CSBD1 at a concentration of 2.0μM did not undergo coacervation under these conditions.

[ELP1-CSBD1] (μM)	Coacervation Temperature (°C)	Rate of Coacervation (Abs units/min)	Maximum Absorbance (°C)
100	29.8 ± 0.5	0.21 ± 0.02	0.97 ± 0.07
50	35.5 ± 1.1	0.09 ± 0.02	0.54 ± 0.09
25	41.0 ± 1.2	0.03 ± 0.003	0.24 ± 0.02
12.5	49.6 ± 2.4	0.006± 0.002	0.08 ± 0.02
6.25	53.5 ± 0.9	0.003 ± 0.001	0.03 ± 0.004
2.0	N/A	N/A	N/A

Compared to the coacervation curves of ELP1 at equivalent concentrations (Bellingham, 2001), ELP1-CSBD1 appears to coacervate at almost the exact same temperatures. For example, Bellingham (2001) determined 25μM ELP1 coacervated at 41.0°C, which is the same value that 25μM ELP1-CSBD1 was found to coacervate.

It has been shown that the overall hydrophobicity of a protein can affect the temperature at which they coacervate (Urry et al., 1991), and since ELP1 and ELP1-CSBD1 have the same hydrophobic regions and relative hydropathy, and differ only in crosslinking domain, it is not entirely surprising that they would coacervate at the same temperature. Previous work by Bellingham et al. (2001) identified that the hydrophobic domains, exons 20 and 24, were necessary for coacervation of ELP1 to occur, as peptides made from the crosslinking domain alone (exons 21 and 23) were incapable of coacervation. The results presented here support this finding, as ELP1-CSBD1 was still able to coacervate despite making significant changes to the crosslinking domain.

Similar to ELP1 (Bellingham et al., 2001), the temperature of coacervation of ELP1-CSBD1 is inversely dependent on polypeptide concentration, as increasing the polypeptide concentration substantially decreases the coacervation temperature. This inverse relationship is

clearly outlined in Figure 11, which also displays a non-linear correlation between peptide concentration and coacervation temperature. This is consistent with previous studies done with tropoelastin (Vrhovski et al., 1997) and ELP1 (Bellingham et al., 2001), which have shown that at lower peptide concentrations, smaller changes in concentration have a larger effect on coacervation temperature compared to similar changes at high peptide concentrations.

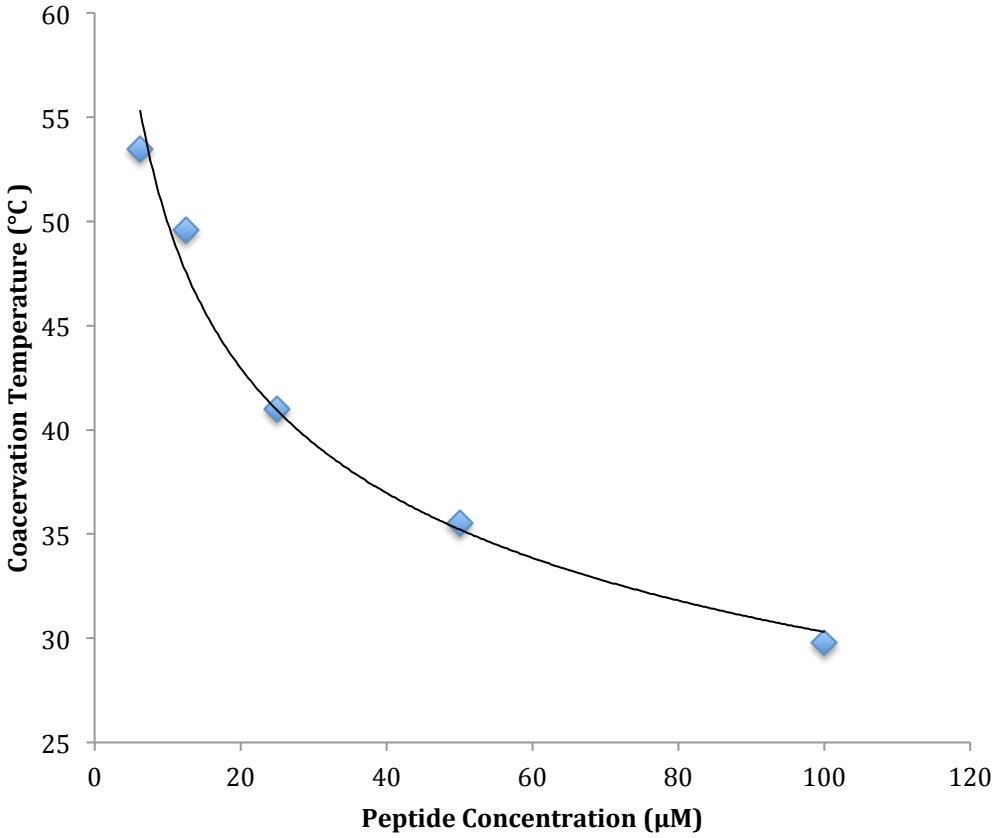


Figure 11: Effect of polypeptide concentration on coacervation temperature of ELP1-CSBD1. Coacervation studies were carried out in standard coacervation buffer (50mM Tris, 1.5M NaCl, 1mM $CaCl_2$) at pH 9.5. Plots of coacervation temperature as a function of polypeptide concentration are shown. A trendline has been added to show the non-linear effect of concentration on coacervation temperature.

The ionic strength of the solution also had a noteworthy effect on coacervation temperature. Initial trials were conducted at a pH of 7.5 and while coacervation did occur, it occurred at approximately 10°C higher than the trials conducted here at a pH of 9.5 (Figure 12).

In addition, both the maximum absorbance reached, as well as the rate of coacervation, were distinctively lower at a pH of 7.5 compared to coacervation studied at pH 9.5. Therefore it can be resolved that at a lower pH, the coacervation of ELP1-CSBD1 is attenuated. Experiments with α-elastin have supported the theory that coacervation is maximal (occurs at the lowest temperature and highest rate) at the isoelectric point when there is no net charge on the protein (Kaibara et al., 1992, Vrhovski et al, 1997). The high isoelectric point of the ELP1-CSBD1 protein (11.17) can be attributed to its positively charged lysine residues found in the binding domain, which differ in number and spacing from the ELP1 polypeptide. Therefore for all other coacervation trials, the coacervation buffer was increased and maintained at a pH of 9.5 in order to compensate for the higher pI of ELP1-CSBD1 compared to that of ELP1, which was the peptide that the initial buffer conditions were based on. The buffer was not increased to the isoelectric point of ELP1-CSBD1 to keep similar conditions to the trials Bellingham et al. (2001) initially conducted using ELP1. Although the ELP1 protein has a pI of 9.81, trials were conducted at pH 7.5 to mimic physiological properties. A similar difference between pH and pI was purposely maintained in these trials using ELP1-CSBD1, which were conducted at pH 9.5 while ELP1-CSBD1 has a pI of 11.17.

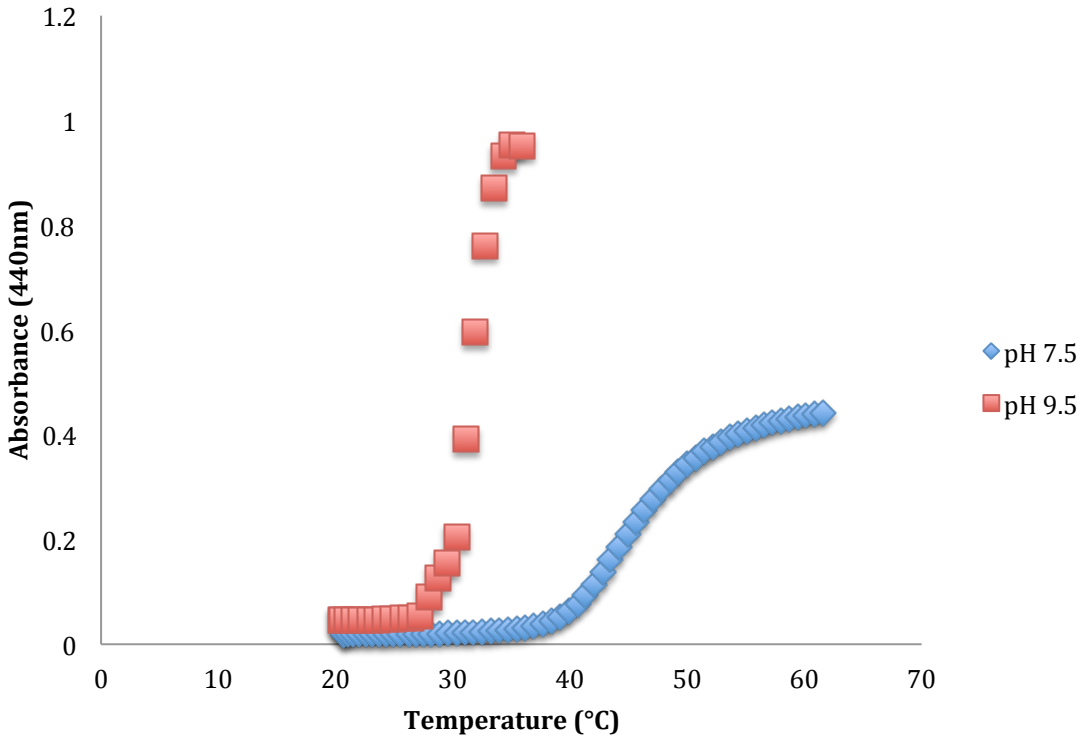


Figure 12: Effect of pH on coacervation temperature of ELP1-CSBD1. Concentration was constant at 100μM and standard coacervation buffer was used in all trials (50mM Tris, 1.5M NaCl, 1mM $CaCl_2$). Six trials at both a pH 7.5 and pH 9.5 were performed. Average coacervation curves for each pH are shown.

The main differences between the coacervation results for ELP1-CSBD1 and that of ELP1 are the rate of coacervation and the maximum absorbance observed. At concentrations of 100μM, ELP1 reached a maximum absorbance of almost 2.0 (Bellingham et al., 2001), whereas at the same concentration of ELP1-CSBD1, as shown in Figure 10, the maximum absorbance reached was only half that at 0.97. Similarly, the rate of coacervation of ELP1-CSBD1 (Table 6) was approximately one fifth of the rate for ELP1 (Bellingham et al., 2001) when comparing it at a concentration of 25μM. It has been suggested that the rate of coacervation may also be related to the nature of the hydrophobic domains (Jensen et al., 2000), however these results challenge this theory, as ELP1 and ELP1-CSBD1 have no variation in hydrophobic domains.

While the importance of hydrophobic domain interactions in the process of coacervation has been widely accepted (Vrhovski et al, 1997; Jensen et al., 2000; Bellingham et al., 2001; Toonkool et al., 2001), there are likely other properties that influence the nature of coacervation. Since the differences in rate and absorbance cannot fully be explained by the number of repeated hydrophobic domains or hydropathy, it can be speculated that the specific nature of the sequences of the crosslinking domains also contribute to the propensity of the ELPs to self-assemble.

ELP1 had a greater difference in amino acid content (Table 4) and molecular weight (Table 2) compared to the ELP1-CSBD1. These subtle differences in amino acid content, or more specifically the amino acid sequence of the crosslinking domains, are also likely to have an effect on the rate and degree of coacervation of ELP1-CSBD1. When looking at the sequence of the CS binding domain, it is much smaller than the crosslinking domain found in ELP1. Additionally, the spacing of the positively charged residues is much different in the binding domain of ELP1-CSBD1 compared to the crosslinking domain of ELP1 in that the lysine residues are arranged considerably closer together (Figure 4). While coacervation will still occur at the same temperature as ELP1, the co-operativity of the aggregation process may have been decreased. These characteristics of the sequence of ELP1-CSBD1 may be hindering the extent of coacervation by slowing the interaction of the hydrophobic domains, and as such the folding of the protein into an aggregate may be occurring at a lower rate. The decrease in absorbance can likely be attributed to the smaller molecular weight of the ELP1-CSBD1 peptide, as smaller aggregates may be forming.

Osborne (2008) was able to visualize the process of coacervation and the formation of coacervate particles using the Woodhouse polypeptide ELP4, a similar polypeptide to ELP1, as shown in Figure 13. The ELP4 was found to spontaneously and immediately self-assemble into

spherical droplets once the coacervation temperature was reached. Once these droplets came into contact, they were observed to coalesce in solution into larger spherical particles.

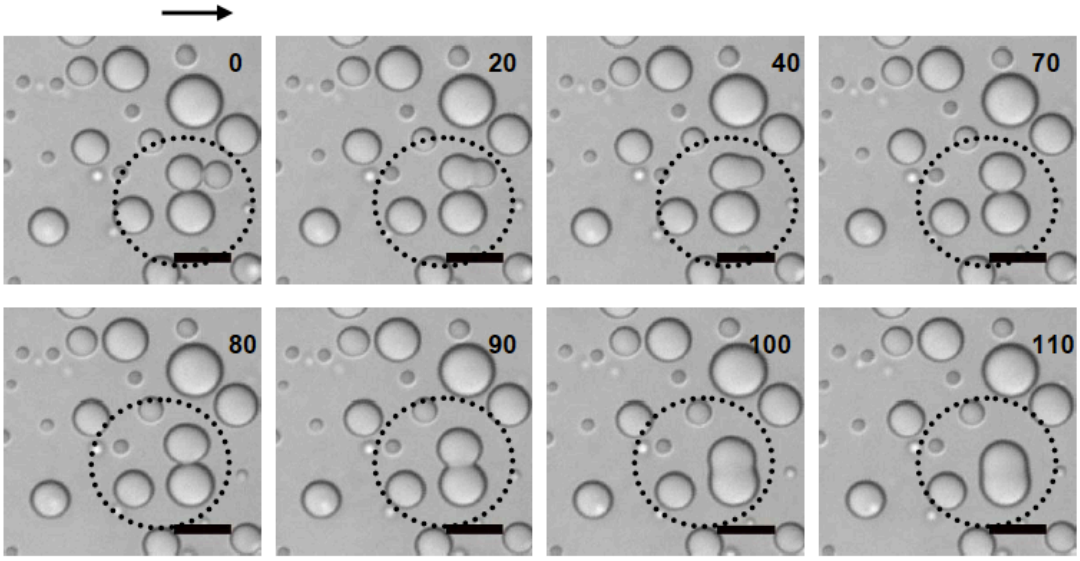


Figure 13: Coalescence of individual ELP4 coacervate particles in solution. Images were taken at multiple time points (indicated in seconds) during the coalescence of ELP4 particles. Scale bar represents 10μm. Reprinted with permission from Woodhouse et al., Self-assembled elastin-like polypeptide particles. Biochemistry (2008) Volume 4 Issue 1. Copyright 2008 Elsevier.

It is anticipated that because of the similarities in coacervation characteristics between the ELP1-CSBD1 and ELP1 polypeptides, and the relationship between ELP1 and ELP4, a similar process is happening to ELP1-CSBD1 as shown in Figure 13. It can be suggested that in a coacervated state, ELP1-CSBD1 droplets form immediately and coalesce with each other in a similar manner. However, as stated above, as the maximum absorbance is much lower for the ELP1-CSBD1 peptide than for ELP1, and as larger ELPs have been shown to reach higher maximum absorbances (Bellingham, 2001) it can be assumed that smaller droplets are forming compared to those shown here.

4.5.1 ELP1-CSBD1/CS Coacervation Studies

As it has been confirmed that replacing the crosslinking domains with binding domains has little effect on the propensity of ELP1-CSBD1 to self-assemble, it is now important to characterize the ELP1-CSBD1 polypeptide in the presence of chondroitin sulphate. GAGs such as CS and heparin have been shown to mediate the coacervation of tropoelastin by promoting aggregate formation (Wu et al., 1999; Tu & Weiss, 2008). However, with the replacement of the typical crosslinking domain of tropoelastin with a CS-specific binding domain, it is likely that the presence of CS will have a different effect on the coacervation of ELP1-CSBD1 compared to basic tropoelastin.

Coacervation studies were conducted in a similar manner to the initial trials with ELP1-CSBD1 on its own. The effects of CS were initially studied at a 1:1 molar ratio with the different concentrations of ELP1-CSBD1. The generated coacervation curves for each ELP1-CSBD1/CS mixture are shown in Figure 14 and are compared to the respective coacervation curves of ELP1-CSBD1 at the same conditions. Quantitative results for each ELP1-CSBD1/CS concentration are summarized in Table 7.

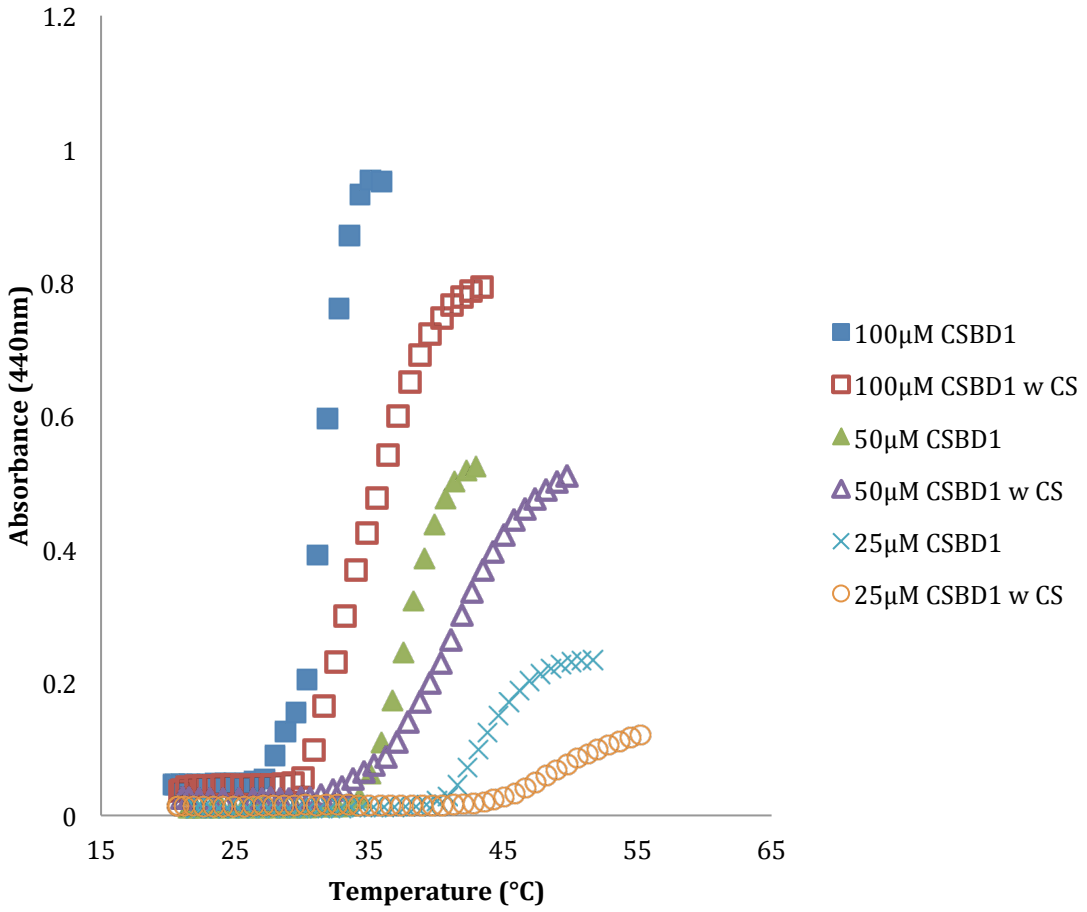


Figure 14: Effect of CS on coacervation temperature of ELP1-CSBD1. Average coacervation curves are shown. Coacervation studies were carried out in standard coacervation buffer (50mM Tris, 1.5M NaCl, 1mM $CaCl_2$) at pH 9.5. Concentrations were varied as indicated, with all other conditions identical. A minimum of 6 coacervation trials was conducted for each concentration.

Table 7: Coacervation temperatures, rates of coacervation and maximum absorbance values corresponding to varying ELP1-CSBD1 concentrations in the presence of CS. Coacervation studies were carried out in standard coacervation buffer (50mM Tris, 1.5M NaCl, 1mM $CaCl_2$) at pH 9.5. A 1:1 ELP1-CSBD1 to CS molar ratio was maintained at each concentration. All values are stated as mean ± standard deviation. A minimum of 6 coacervation trials was conducted for each concentration.

[ELP1-CSBD1] (µM)	[CS] (µM)	Coacervation Temperature (°C)	Rate of Coacervation (Abs units/min)	Maximum Absorbance (°C)
100	100	32.3 ± 0.8^{1}	0.11 ± 0.01^{1}	0.82 ± 0.09^{1}
50	50	37.7 ± 2.4	0.06 ± 0.02^{2}	0.57 ± 0.08
25	25	45.2 ± 3.3^{2}	0.01 ± 0.01^{1}	0.16 ± 0.05^{1}

Significantly different from respective trials of ELP1-CSBD1 on its own: 1. $p<0.01$, 2. $p<0.025$

As expected, ELP1-CSBD1 was able to undergo coacervation at all three concentrations, suggesting that ELP1-CSBD1 is still able to self-assemble in the presence of CS. This supports findings by Velebny et al. (1981), Wu et al. (1999), and Tu and Weiss (2008) whom have all shown that tropoelastin aggregates upon exposure to chondroitin sulphate. However, it is apparent that exposure to CS has a much different effect on the temperature, rate, and maximum absorbance of coacervation of ELP1-CSBD1 compared to ELP1.

Previous studies have shown that that addition of CS to tropoelastin significantly lowers the coacervation temperature (Wu et al., 1999; Tu & Weiss, 2008). However, the addition of CS to the ELP1-CSBD1 polypeptide had the opposite effect, as the coacervation temperature was increased at all concentrations (Table 7). These results are important as they indicate that there must be an underlying reason specific to ELP1-CSBD1 causing this shift in coacervation temperature. It is speculated that this is a result of interactions between the CS and the ELP1-CSBD1 peptide. As the binding domain in the ELP1-CSBD1 is specific for chondroitin sulphate, these coacervation results suggest that perhaps binding is occurring between these molecules. If in fact CS were binding to ELP1-CSBD1, it would indeed be more difficult for the peptide to undergo coacervation. The presence of a CS molecule binding to the ELP1-CSBD1 molecule would further hinder the interaction of the hydrophobic domains of the polypeptide. Even if the hydrogen bonds in the clathrate structure surrounding the ELP were disrupted by an increase in temperature, the bulky CS molecules that are interacting with the binding domains could potentially deter the hydrophobic domains of the elastin to interact and assume a folded shape. In addition, CS is extremely hygroscopic and strongly attracts and holds water molecules from the surrounding environment (Ebube et al., 2002). Therefore, if CS were in fact binding to ELP1-CSBD1, its hygroscopic properties would likely deter the disruption of the water clathrate

structure surrounding the ELP1-CSBD1 molecules in solution and as such, not allow coacervation to occur. The results of the ELP1-CSBD1/CS coacervation trials also show a statistically significant decrease in both the rate of coacervation and the maximum absorbance observed. This further supports the theory that CS may be binding to the ELP1-CSBD1 peptide, reducing the overall co-operativity of coacervation.

It is assumed that this binding event would involve an ionic interaction between the positively charged residues in the binding domain of ELP1-CSBD1 and the negatively charged sulphates of the CS. This is a reasonable assumption based on our objectives as well through studies of other GAG-protein interactions. For example, in the absence of GAGs, it was found that blocking lysine residues had no major effect on coacervation, but in the presence of GAGs coacervation was highly attenuated when lysine chains were blocked (Wu et al., 1999). Additionally, it has been predicted that in order to interact with a linear GAG, any positively charged residues would have to line up along the same side of the protein segment (Esko et al., 2009). Therefore, since the ELP1-CSBD1 binding domain contains closely spaced positive lysine residues, it would be reasonable to assume interaction between this peptide and CS would occur. Additionally, the binding domain sequence used in ELP1-CSBD1 has already been determined to bind to CS (A. Panitch, personal communication). The specificity underlying this particular reaction, however, cannot be elucidated until further studies of this interaction are conducted.

4.5.2 ELP1-CSBD1/Heparin Coacervation Studies

While the CSBD sequences were selected for against CS, it was found that they exhibited stronger heparin binding than CS binding (A. Panitch, personal communication, May 23, 2012). Heparin and the closely related heparan sulphate (HS) are complex, highly sulphated GAGs that have a number of important biological properties. They are one of the most studied groups of

GAGs in association to vascular development and response, and heparin is widely used as an anticoagulant (Capila & Linhardt, 2002; Lodish et al., 2003). The polysaccharide chains of heparin vary in length and chemical structure, but are primarily composed of alternating uronic acid and glucosamine residues (Fromm et al., 1997), shown in Figure 15.

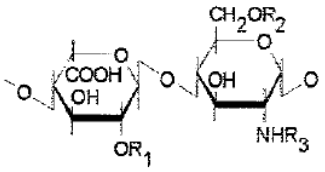


Figure 15: Generalized diagram of a heparin disaccharide: $R_1 = SO_3^-$ or H; $R_2 = SO_3^-$ or H; $R_3 = SO_3^-$ or $COCH_3$. Reprinted and adapted with permission from Mulloy and Forster, Conformation and Dynamics of Heparin and Heparan Sulfate. Glycobiology (2000) Volume 10 Issue 11. Copyright © 2000, Oxford University Press.

Heparin has been shown to play a key role in many vascular events. In terms of angiogenesis, heparin has been associated with the formation of new blood vessels, and ultrastructural evidence has shown significant amounts of heparan sulphate in capillaries (Castellot et al 1986). Heparin has also been found to influence proliferation and attachment of vascular wall components (Orlidge & D'Amore, 1986; Tan et al., 1989).

Following vascular injury, heparin has been associated with various natural repair mechanisms. Therapeutically, heparin has been used as an anticlotting drug due to its ability to activate the natural clotting inhibitor antithrombin III (Lodish et al., 2003). Tan et al. (1989) found that following experimental arterial injury *in vivo*, heparin suppresses smooth muscle cell proliferation and inhibits intimal hyperplasia. It was also found that heparin influences the deposition of ECM in blood vessels following injury (Tan et al, 1989). Heparin is also known to stimulate the growth and activity of ECs (Dahlbäck, 2000; Heyligers, 2005). Due to the prevalence of heparin in vascular injury and repair, it would be beneficial to look into the potential of this GAG in TEBV applications.

Building on the potential binding abilities of heparin to ELP1-CSBD1 as well as the known vascular repair properties of heparin, coacervation studies were also conducted on ELP1-CSBD1 in the presence of heparin. Trials were carried out in standard coacervation buffer (50mM Tris, 1.5M NaCl, 1mM $CaCl_2$) and at polypeptide concentrations of 100μM, 50μM, and 25μM. Heparin was added at a 1:1 molar ratio with the different concentrations of ELP1-CSBD1. The generated coacervation curves for each ELP1-CSBD1/heparin mixture are shown in Figure 16 and are compared to the respective coacervation curves of ELP1-CSBD1 at the same conditions. Quantitative results for each ELP1-CSBD1/heparin concentration are summarized in Table 8.

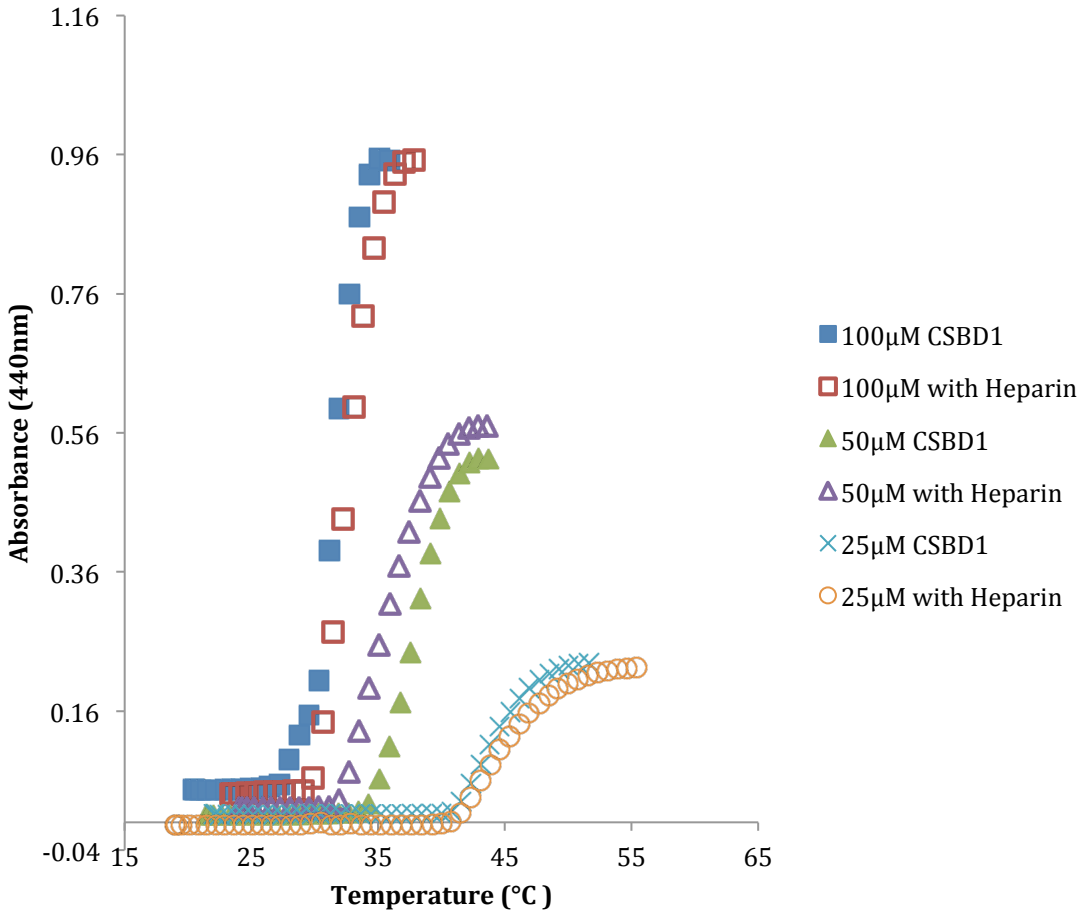


Figure 16: Effect of heparin on the coacervation of ELP1-CSBD1. Average coacervation curves are shown. Coacervation studies were carried out in standard coacervation buffer (50mM Tris, 1.5M NaCl, 1mM $CaCl_2$) at pH 9.5. Concentrations were varied as indicated, with all other conditions identical. A total of 6 trials were completed for ELP1-CSBD1/heparin concentrations of 100µM and 50µM while 3 trials were completed using 25µM.

Table 8: Coacervation temperatures, rates of coacervation and maximum absorbance values corresponding to varying ELP1-CSBD1 concentrations in the presence of heparin. Coacervation studies were carried out in standard coacervation buffer (50mM Tris, 1.5M NaCl, 1mM $CaCl_2$) at pH 9.5. A 1:1 ELP1-CSBD1 to HS molar ratio was maintained at each concentration. All values are stated as mean ± standard deviation.

[ELP1-CSBD1] (μM)	[HS] (μM)	Coacervation Temperature (°C)	Rate of Coacervation (Abs units/min)	Maximum Absorbance (°C)
100	100	$30.7 \pm 0.5^{1,3}$	0.18 ± 0.03^{3}	0.98 ± 0.09^{4}
50	50	33.9 ± 2.1^{4}	0.09 ± 0.03	0.61 ± 0.16
25	25	40.9 ± 0.4	$0.02 \pm 0.004^{2,3}$	0.22 ± 0.01

Significantly different from respective trials of ELP1-CSBD1 on its own: 1. $p<0.01$, 2. $p<0.025$
Significantly different from respective trials of ELP1-CSBD1 in presence of CS: 3. $p<0.01$, 4. $p<0.025$

Similar to the previous studies conducted, ELP1-CSBD1 was also able to coacervate when exposed to heparin. However, when in the presence of heparin, coacervation of ELP1-CSBD1 did not appear to be significantly impacted relative to the coacervation of ELP1-CSBD1 on its own. Weiss et al. (1999, 2008) have shown that heparin displaces coacervation of synthetic human tropoelastin to lower temperatures. The current study indicates that while there may be a slight change in coacervation temperature of ELP1-CSBD1 in the presence of heparin, the results are not statistically significant. This difference between the effect of heparin on a synthetic ELP and on ELP1-CSBD1 could potentially be attributed to the change in crosslinking domains. The interaction between the negative charges of heparin and the positive charges on the tropoelastin molecule are thought to neutralize repulsion and therefore make it easier for tropoelastin molecules to interact (Vrhovski et al., 1997; Wu et al., 1999). Therefore, it can be speculated that interaction between the binding domain of ELP1-CSBD1 and heparin is not strong enough to decrease that repulsion between molecules and coacervation occurs to the same extent as ELP1-CSBD1 on its own.

It is also apparent that there are disparities between the coacervation of ELP1-CSBD1 in the presence of heparin and in the presence of CS. Heparin and chondroitin sulphate differ in their galactosamine content and display different charge densities, with heparin having the

highest negative charge density of any molecule in the body (Wu et al., 1999), which would explain their differences in interaction with the ELP1-CSBD1 peptide. Wu et al. (1999) have suggested that different coacervation curve patterns between CS and heparin may be due to their different charge densities and distributions between the two. A good depiction of this is in Figures 17 and 18, which display a large variation between the curve shapes of ELP1-CSBD1 exposed to CS and exposed to heparin.

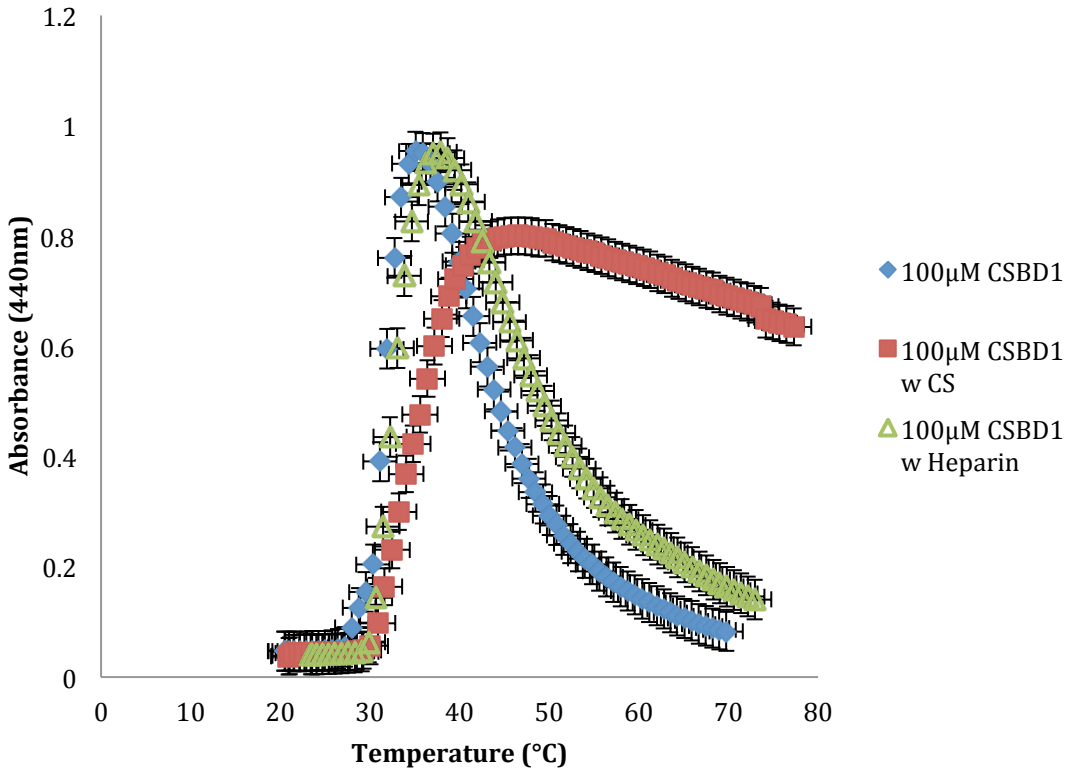


Figure 17: Coacervation of 100µM ELP1-CSBD1 alone and in the presence of CS and heparin. Average coacervation curves are shown. Coacervation studies were carried out in standard coacervation buffer (50mM Tris, 1.5M NaCl, 1mM $CaCl_2$) at pH 9.5. There is a statistically significant difference between all parameters of ELP1-CSBD1/CS coacervation vs ELP1-CSBD1 and CSBD/heparin. It is important to note the difference in curve shape in the presence of CS versus the other two conditions.

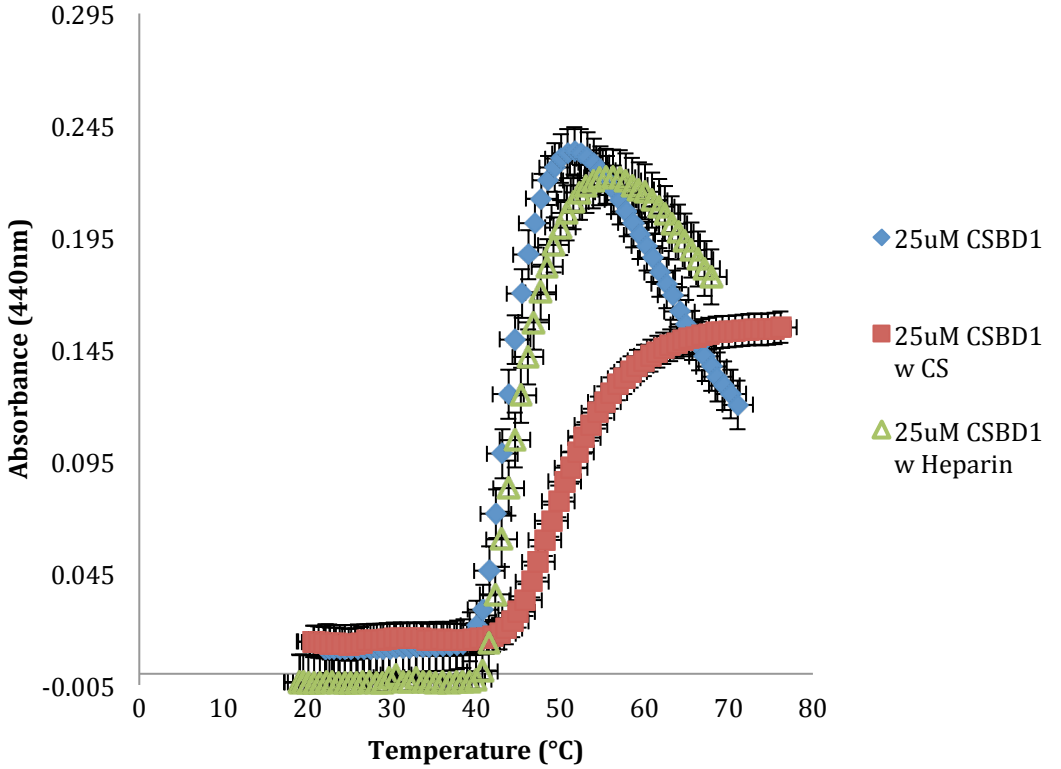


Figure 18: Coacervation of 25μM ELP1-CSBD1 alone and in the presence of CS and heparin. Average coacervation curves are shown. Coacervation studies were carried out in standard coacervation buffer (50mM Tris, 1.5M NaCl, 1mM $CaCl_2$) at pH 9.5. There is a statistically significant difference between ELP1-CSBD1/CS coacervation vs ELP1-CSBD1 coacervation. It is important to note the difference in curve shape in the presence of CS versus the other two conditions.

Looking at these figures it appears that at higher temperatures aggregation is only slightly attenuated when in ELP1-CSBD1 is in the presence of CS, whereas when alone or with heparin, absorbance rapidly decreases again. This further supports the theory that there is a weak interaction between heparin and ELP1-CSBD1. It can then also be speculated that there may be a strong interaction occurring between ELP1-CSBD1 and CS.

As it has been well established that the hydrophobic regions play a major role in the coacervation of elastin and elastin-like polypeptides, it is likely that both CS and heparin have a significant effect on these regions as well. Panitch et al. (2010) have suggested that hydrophobic

forces may be more significant for CS binding than for heparin binding, attributing it to the fact that CS is much less sulphated than heparin. This could again explain the difference in coacervation between CS and HS and suggest that a stronger interaction is occurring between ELP1-CSBD1 and CS.

The helical structure of the crosslinking domains has also been shown to be an important factor in the coacervation of elastin, and could also be affected by the presence of GAGs. Studies have shown that the coacervation temperature of tropoelastin decreases linearly with increasing helical structure (Muiznieks, et al., 2003), and Yeo et al. (2011) have suggested that the presence of GAGs support this helical formation in the crosslinking domains. There is reason to believe then, that the differences in interaction between ELP1-CSBD1 and CS and heparin could potentially be related to variations in helix formation, likely attributed to differences in charge densities and interactions with the hydrophobic domain.

To ensure that this interaction with CS is specific to ELP1-CSBD1, coacervation of ELP1 in the presence of CS was studied. Trials at a 1:1 ELP1 to CS ratio of concentrations of 100μM were conducted, as there appeared to be the greatest effect on coacervation at this concentration. The results were relatively consistent with those found by Bellingham et al. (2001) for coacervation of ELP1 on its own. A comparison of the coacervation of ELP1 in the presence of CS and of ELP1-CSBD1 in the presence of CS is shown in Figure 19. The absorbance of ELP1 in the presence of CS was much higher than ELP1-CSBD1 in the presence of CS, with a maximum value of approximately 1.3 achieved versus 0.82, respectively. This is closer to the maximum absorbance of ELP1 coacervation, which reached a maximum absorbance of almost 1.8. In terms of the rate of coacervation, it was much higher for ELP1 than ELP1-CSBD1 when they were exposed to CS. The average rate of coacervation for 100μM ELP1 with CS was found

to be 0.42 Abs units/minute, which is more than double the rate found for ELP1-CSBD1 with CS, a value of 0.18 Abs units/minute. While still lower than the rate determined for 100µM ELP1, this value is much closer to the rate Bellingham (2001) determined that was approximately 0.8 Abs units/minute.

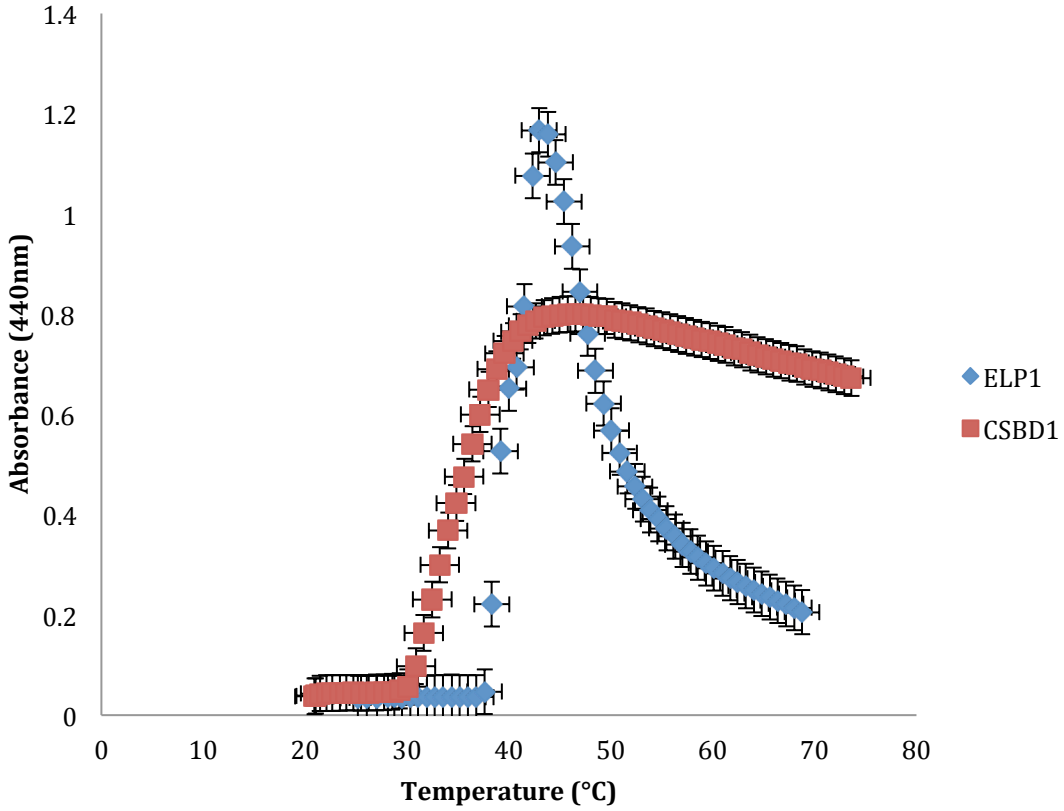


Figure 19: Coacervation of 100µM ELP1 in the presence of CS vs. 100µM ELP1-CSBD1 in the presence of CS. Coacervation studies were carried out in standard coacervation buffer (50mM Tris, 1.5M NaCl, 1mM $CaCl_2$) at pH 9.5. Average coacervation curves are shown for four and six trials of each condition, respectively.

There did appear to be a major variance between the coacervation temperature of ELP1 and ELP1-CSBD1 in the presence of CS. While 100µM ELP1-CSBD1 was found to coacervate at approximately 32°C when exposed to CS, 100µM ELP1 in the presence of CS coacervated at a

much higher average temperature of 39.4°C. This is also much different than 100μM ELP1 on its own, which was found to coacervate at approximately 30°C (Bellingham et al., 2001).

These results are in contrast to the studies done by Wu et al. (1999) and Tu & Weiss (2008) who, as previously discussed, determined that in the presence of CS their synthetic elastin-based polypeptides coacervated at lower temperatures. However, it is important to note that these trials were completed at much higher protein concentrations than the studies conducted in this paper, and the ratios of GAG to ELP were not 1:1. Additionally, it is likely that the polypeptides varied slightly in structure and composition, as the synthetic peptides were prepared in separate labs.

It is very important to point out that while ELP1 in the presence of CS coacervated at a much higher temperature than ELP1 on its own, the overall shapes of the coacervation curves are very similar in that once a maximum absorbance is reached, the absorbance rapidly begins to decrease until it reaches absorbance values similar to those found before coacervation occurred. This is different compared to ELP1-CSBD1 in the presence of CS, which as previously shown, does not rapidly decrease and aggregates are still apparent at higher temperatures. Therefore, the differences in absorbance after coacervation could again be indicative of a different interaction occurring between CS and the ELP1-CSBD1 peptide versus the interaction between CS and ELP1.

Chapter 5

Conclusions and Recommendations

5.1 Summary

The development of small-diameter blood vessels that mimic the properties of natural vessels has proven to be a clinical challenge. Whereas synthetic materials have been successful in larger applications, these solutions have been unsuccessful in small-caliber environments due to a number of factors including thrombus formation, intimal hyperplasia, and infection (Isenberg et al., 2006). A variety of solutions have been proposed, with the most recent focus on biomimetic strategies that will imitate the basic behavior and functions of native vasculature. While this approach is promising, there are still many limitations on designing a tissue-engineered blood vessel that successfully replicates natural vessel properties. One major limitation is effectively replicating the properties of the intimal/medial layer of the vessel by providing a graft that can effectively modulate SMC response, thus inhibiting the formation of intimal hyperplasia. Seeding smooth muscle cells onto graft surfaces has been attempted, but has been faced with many challenges including shedding of the cell layer and inducing unwanted immunogenic responses. In order to address these issues, recent evidence has suggested that certain engineered vessels may have the ability to provide SMC mediating abilities through the incorporation of ECM materials that are involved in the vascular wound healing response.

This work focuses on the development of a new polymer that has the potential to function as a component of a small-diameter blood vessel that can modulate SMC proliferation and migration. Using recombinant elastin-like polypeptides developed by the Woodhouse laboratory, as well as chondroitin sulphate-specific binding sequences elucidated by the Panitch laboratory, a

new elastin-like polypeptide-chondroitin sulphate binding domain block copolymer has been developed and characterized. It is anticipated that when combined with the glycosaminoglycan chondroitin sulphate, this polypeptide will gel, forming a basis for a intimal/medial layer of a TEBV that will modulate SMC response and provide the necessary mechanical integrity to the graft while preserving its elastic properties.

The expression of ELP1-CSBD1 and ELP1-CSBD2 has been demonstrated in an *E. coli* system. The polypeptides were purified using dialysis and ion exchange chromatography and characterization was completed using mass spectrometry and amino acid analysis to assess expression and purity. ELP1-CSBD1 was successfully expressed using these methods and produced to high purities. ELP1-CSBD2 was also successfully produced using the same expression system, but not yet to high purities.

Coacervation was used to determine the self-assembly characteristics of ELP1-CSBD1, as well as to investigate the influence of chondroitin sulphate on the propensity of ELP1-CSBD1 to self-aggregate. ELP1-CSBD1 was able to undergo coacervation *in vitro,* suggesting that ELP1-CSBD1 is able to self-assemble similar to native elastin. Additionally, this study showed that in the presence of CS, the coacervation characteristics of ELP1-CSBD1 are different than when the peptide is on its own. Specifically, the coacervation temperature is increased, the rate and extent of coacervation is decreased, and aggregates remain in solution at higher temperatures. The influence of heparin was also explored as previous studies indicated that the CS binding domains also bind to heparin. The current study indicates that while there was a slight change in coacervation characteristics of ELP1-CSBD1 in the presence of heparin, the results are not statistically significant to assume that heparin has a substantial effect.

These results suggest that there is some type of interaction occurring between the ELP1-CSBD1 and CS. While it cannot be confirmed that binding is occurring between these molecules, it is a likely explanation based on previous studies that have showed successful binding between these CS binding sequences and CS. However, further investigations into the mechanisms driving this behaviour are required.

To the author's knowledge, the current work is the first reported study to successfully incorporate chondroitin sulphate binding domains into an elastin-like polypeptide. This is significant in proving the ability to manipulate the crosslinking regions of elastin-like polypeptides to bind to GAGs while maintaining properties similar to native elastin.

5.2 Recommendations and Future Work

While the results provided in this study indicate that the interactions with CS appear to be occurring with the ELP1-CSBD1 polymer, there is a need to characterize these polypeptides further. This includes investigating their structural properties, other interactions with the GAGs and if gelation will occur in their presence, and if these polypeptides could modify SMC behaviour when included as a component of an intimal/medial layer in a tissue-engineered blood vessel.

Based on the current findings, it will be necessary to further investigate other ways of obtaining high purities of the ELP1-CSBD2 protein, and perhaps even to increase the purity of ELP1-CSBD1. There does not seem to be a problem with the expression of the proteins, but in case, phage contamination was evaluated during the fermentation process and it appeared that no contamination was occurring. Therefore, it will be important to evaluate the purification strategies used to purify the CSBD peptides. It may be beneficial to look at using different buffer solutions during purification using ion exchange chromatography, or perhaps even using a

different purification method itself. A sodium acetate buffer at pH 5 was used in preparation for purification on an ion exchange column, but changing the buffer pH and even buffer composition would likely improve purity of the protein. Because the pI of the ELP1-CSBDs is so high, purification in a buffer with a higher pH would likely get rid of more contaminants when loading the extract. For example, the use of a HEPES buffer at pH 7.5 has been shown to improve purities (J. Rouleau, Elastin Specialties, personal communication).

It may also be beneficial to look at purification methods other than ion exchange chromatography when purifying the proteins. These protein constructs are all designed as GST fusion proteins and thus have the ability to be purified using a glutathione affinity column, which could perhaps enhance purification of the proteins. Additionally, as the ability of these proteins to aggregate under specific temperature or ionic strength has been proven under specific conditions, this could potentially provide a simple method of purification. Meyer and Chilkoti (1999) termed this approach inverse transition cycling (ITC). It involves triggering the phase transition of an ELP by addition of heat or salt, followed by separation of the aggregated ELP from solution by centrifugation, then finally resolubilization in cold buffer and one final round of centrifugation to eliminate contamination. This is considered to be one full cycle of ITC and can be repeated several times in order to increase the purity of the ELP protein. It is anticipated that continued investigations into purification methods would result in improving the efficiency of purification and would increase the final purities achieved.

The effect of replacing the crosslinking domains of an elastin-like polypeptide with the other CS binding sequences will also need to be further investigated. There were three different binding sequences proposed by the Panitch laboratory, and it is anticipated that the differences in sequence may have an effect on the binding event, currently unknown within an EP backbone.

Coacervation using these other binding sequences will also be of interest to determine the sequence effect on self-assembly *in vitro*. In addition, it will be interesting to investigate the modification of the other ELPs designed in the Woodhouse laboratory (ELP2 and ELP4) with the binding sequences. It is assumed that an increase in the length of the polypeptide and number of chondroitin sulphate binding domains would enhance the capacity of the polypeptide to bind to CS and would also increase the mechanical strength of the materials.

One of the most significant properties of these ELP1-CSBDs that will need to be investigated is their ability to bind to and gel in the presence CS. To evaluate this, a variety of methods could be used. In Vrhovski's work (1997), she quantified the binding of heparin to tropoelastin using Scatchard analyses according to Lee and Lander's method. More recent research typically analyzes the dissociation constant between molecules to determine the extent of binding. Jeong et al. (2008) have designed a novel assay that was used to probe heparin-peptide interactions using pentapeptide-stabilized gold nanoparticles, which could potentially be applied to this application as well. Recent research has focused on immobilization of proteins on biochips to analyze binding (Tu & Weiss, 2008; Rusmini et al., 2007). If gelation does occur as anticipated, a capillary remoter or a dynamic mechanical analyzer could be used to provide an analysis of the mechanical properties of the gel including shear and compressive properties, which would be important to quantify for its use in blood vessel applications.

Structural studies would also be beneficial to perform in order to elucidate the mechanisms behind binding and coacervation of the ELP1-CSBDs. Circular dichroism could be used to observe conformational changes of the peptides during coacervation. In addition, light scanning electron microscopy (SEM), as used by Osborne (2008), would be beneficial to use to look at the structure of the coacervates. When looking at binding between the ELP1-CSBDs and

CS or other GAGs, Zamfir et al. have suggested the use of capillary electrophoresis in combination with electrospray ionization mass spectrometry (ESI-MS) as a method that can provide information about the structure of GAG-based molecules.

Studies into the effect of the ELP1-CSBD/CS gels on SMC proliferation and migration are also of great interest. To investigate the behaviour of SMCs in constructs made with the ELP1-CSBD/CS gels, an *in vitro* SMC culture system can be employed. In order to analyze the adhesion and proliferation properties of the SMCs, SEM and confocal microscopy can be used, and a cell proliferation assay kit could be utilized to evaluate the proliferation of the SMCs.

Finally, it would be advantageous to investigate the use of other GAG binding domains incorporated into an ELP backbone. For example, because heparin did not appear to show significant binding in this study, it would be beneficial to look at other domain sequences that could potentially bind to heparin. Heparin has been shown to inhibit SMC proliferation, reduce SMC migration, and alter the production of ECM components by SMCs (Clowes & Karnovsky, 1977; Snow et al., 1990). Therefore, it would be beneficial to determine a heparin-binding domain (HBD) that could be incorporated into an ELP to form an ELP-HBD that had the potential to be used in similar applications as an ELP-CSBD.

In conclusion, the results presented in this thesis are significant in that it is the first reported study to successfully incorporate chondroitin sulphate binding domains into an elastin-like polypeptide. This is noteworthy in that it proves that the crosslinking regions of ELPs can be manipulated to include a binding region that has the ability to bind to chondroitin sulphate while maintaining similar properties to native elastin. These results prove there is little effect on the

propensity for self-assembly when replacing the crosslinking domains with ELP1-CSBDs. While the nature of the interaction between the ELP1-CSBDs and CS has yet to be confirmed, based on previous research, it can be assumed that binding is likely occurring. These ELP-CSBD polypeptides have significant potential in many clinical areas where other synthetic polymers have failed, including in small-diameter applications as a component of a composite TEBV. It is anticipated that incorporation of the ELP-CSBD polypeptides in a TEBV as part of an intimal/medial component will improve the patency of small-diameter bypass grafts in the peripheral circulation by increasing the elastic properties as well as modulating SMC response, thereby reducing the formation of intimal hyperplasia.

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Appendix A

Transformation of E. coli with the Recombinant ELP1-CSBD1 Vector

Blast-Align Analysis of Sequencing Results from Transformed E. coli

```
Query = Theoretical ELP1-CSBD1 sequence
Sbjct = Full sequencing results from ACGT Corp

Score =  538 bits (291),  Expect = 2e-157
 Identities = 291/291 (100%), Gaps = 0/291 (0%)
 Strand=Plus/Plus

Query  1
ATGCCGGGCTTTGGTGTCGGAGTCGGAGGTATCCCTGGAGTCGCAGGTGTCCCTGGTGTC  60

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19
ATGCCGGGCTTTGGTGTCGGAGTCGGAGGTATCCCTGGAGTCGCAGGTGTCCCTGGTGTC  78

Query  61
GGAGGTGTTCCCGGAGTCGGAGGTGTCCCGGGAGTTGGCATTTCCCCCATTAAATTTATT  120

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79
GGAGGTGTTCCCGGAGTCGGAGGTGTCCCGGGAGTTGGCATTTCCCCCATTAAATTTATT  138

Query  121
CATCGCAAATATCGCAAACATGGCTTAGTTCCTGGTGTCGGCGTGGCTCCTGGAGTTGGC  180

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  139
CATCGCAAATATCGCAAACATGGCTTAGTTCCTGGTGTCGGCGTGGCTCCTGGAGTTGGC  198

Query  181
GTGGCTCCTGGTGTCGGTGtggctcctggagttggcttggctcctggagttggcgtggct  240

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  199
GTGGCTCCTGGTGTCGGTGTGGCTCCTGGAGTTGGCTTGGCTCCTGGAGTTGGCGTGGCT  258

Query  241  cctggagttggtgtggctcctggcgttggcgtggctCCCGGCATTGGCCCG  291
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  259  CCTGGAGTTGGTGTGGCTCCTGGCGTTGGCGTGGCTCCCGGCATTGGCCCG  309
```

Appendix B

Expression and Purification of ELP1-CSBD1

ELP1-CSBD Expression – Fermentation Results

Figure 20: Results of ELP1-CSBD1 fermentations. Absorbance at 600nm was monitored every hour. Average results for all trials are shown. Cell pellet yields for each trial and overall average are also shown.

CSBD1	Absorbance				
Elapsed Time (h)	Trial 1	Trial 2	Trial 3	Trial 4	AVERAGE
0	0.25	0.18	0.14	0.00	0.1
1	0.23	0.21	0.24	0.08	0.2
2	0.45	0.24	0.37	0.07	0.3
3	0.59	0.69	0.91	0.16	0.6
4	1.79	2.35	3.21	0.98	2.1
5	2.92	4.63	7.33	6.72	5.4
6	8.11	6.97	9.20	8.66	8.2
7	8.30	8.00	10.30	11.55	9.5
8	8.97	8.03	13.57	10.95	10.4
Cell Pellet Yields (g)	212	144	161	169	172

Figure 21: Results of ELP1-CSBD2 fermentations. Absorbance at 600nm was monitored every hour. Average results for all trials are shown. Cell pellet yields for each trial and overall average are also shown.

CSBD2	Absorbance				
Elapsed Time (h)	Trial 1	Trial 2	Trial 3	Trial 4	AVERAGE
0	0.18	0.08	0.27	0.17	0.2
1	0.35	0.41	0.26	0.38	0.3
2	0.30	0.85	0.49	0.33	0.5
3	0.57	2.00	0.85	0.72	1.0
4	1.28	5.94	1.53	1.20	2.5
5	2.90	7.96	3.36	1.47	3.9
6	5.68	9.73	5.59	5.05	6.5
7	7.84	11.2	10.0	9.15	9.5
8	8.30	13.0	9.5		10.3

Amino Acid Analysis Spreadsheet - Sample

AMINO ACID ANALYSIS

CSBD1
SAMPLE: R2.1 -A
Date 16-Jul-12

LOAD VOL(uL): 4.0
MOL. WT.: 8457

							MOLAR RATIO				
	PMOLES	NMOLES	RES WT	NGRAMS	RES/1000	RES/MOLE	EXPEC	Working	OBS		
ASP	1120	1.12	115.09	128.9	6.6	0.7	**0**	**0.0**	**0.6**	0.0000	0.0000
GLU+GLN	1494	1.49	257.24	192.8	8.8	1.0	**0**	**0.0**	**0.8**	0.0000	0.0000
SER	1740	1.74	87.08	151.5	10.3	1.1	**1**	**87.1**	**1.0**	0.0144	0.0001
GLY	56308	56.31	57.05	3215.2	332.3	36.8	**32**	**1,825.6**	**31.9**	0.0031	0.0010
HIS	4221	4.22	137.14	579.2	24.9	2.8	**2**	**274.3**	**2.4**	0.1959	0.0049
ARG	5018	5.02	156.19	783.8	29.6	3.3	**2**	**312.4**	**2.8**	0.4214	0.0125
THR	598	0.60	101.11	60.5	3.5	0.4	**0**	**0.0**	**0.3**	0.0000	0.0000
ALA	14428	14.43	71.09	1025.8	85.1	9.4	**8**	**568.7**	**8.2**	0.0218	0.0019
PRO	26455	26.45	97.12	2568.8	156.1	17.3	**15**	**1,456.8**	**15.0**	0.0008	0.0001
TYR	1955	1.96	163.18	319.1	11.5	1.3	**1**	**163.2**	**1.1**	0.1076	0.0012
VAL	35749	35.75	99.14	3542.8	211.0	23.4	**23**	**2,280.2**	**20.3**	0.1194	0.0252
MET	169	0.17	131.19	22.2	1.0	0.1	**0**	**0.0**	**0.1**	0.0000	0.0000
CYS	0	0.00	103.15	0.0	0.0	0.0	**0**	**0.0**	**0.0**	0.0000	0.0000
ILE	7928	7.93	113.16	897.4	46.8	5.2	**5**	**565.8**	**4.5**	0.1017	0.0048
LEU	3878	3.88	113.16	438.9	22.9	2.5	**2**	**226.3**	**2.2**	0.0984	0.0023
PHE	3535	3.53	147.18	520.3	20.9	2.3	**2**	**294.4**	**2.0**	0.0013	0.0000
LYS	4848	4.85	128.17	621.6	28.6	3.2	**3**	**384.5**	**2.7**	0.0843	0.0024
TOTALS		169.44		15068.7	1000.0	110.7	96	8,457	**96.0**	**Purity (%)**	94.4