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## Controlled Delivery of Serp-1 Protein from Poly(vinyl alcohol) Hydrogel

Karen L. Kennedy, *The University of Western Ontario*

Supervisor: Dr. Wankei Wan, *The University of Western Ontario*

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

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# **CONTROLLED DELIVERY OF SERP-1 PROTEIN FROM POLY(VINYL ALCOHOL) HYDROGEL**

(Spine title: Controlled Protein Delivery from Poly(vinyl alcohol) Hydrogel)

(Thesis format: Monograph)

by

**Karen L. Kennedy**

Biomedical Engineering Graduate Program

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

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

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THE UNIVERSITY OF WESTERN ONTARIO  
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

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entitled:

**Controlled Delivery of Serp-1 Protein from  
Poly(vinyl alcohol) Hydrogel**

is accepted in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

Date \_\_\_\_\_

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## ABSTRACT

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Poly(vinyl alcohol) (PVA) was selected and evaluated as a controlled drug delivery matrix for Serp-1, a potential new therapeutic with anti-inflammatory properties for control of restenosis. PVA hydrogels, containing a high water content, can be formed by physical crosslinking via a process involving freezing and thawing the material in multiple cycles. PVA, being a well known biomaterial, is suited for biomedical applications and the high water content and hydrophilicity provides a friendly environment for the delivery of large protein based drugs. Using bovine serum albumin (BSA) as a model protein, the controlled release properties of PVA were investigated. Release profiles demonstrated diffusion controlled release and adjusting the number of freeze-thaw cycles resulted in a change in release rate. It was also determined that fabricating a reservoir-type system by adding an additional layer of protein-free PVA as an outer barrier provides a method of changing the release kinetics. From a one-layer matrix-type PVA/BSA system the release kinetics follow diffusion controlled release, while from a two-layer reservoir-type system the release kinetics are zero-order. The thickness of the outer barrier controlled the rate of BSA release. Using BSA as a model protein again, its release from PVA hydrogel into phosphate buffered saline (PBS) solutions of varying ionic strength was explored. The amount of BSA released was shown to be influenced by the ionic strength of PBS used as the release medium. Increasing the ionic strength of the release medium caused a reduction in mesh space of the hydrogel and increase in apparent PVA concentration, leading to a reduction in BSA release. These results are important to consider for applications in the physiological environment where salts are unavoidable.

The release of Serp-1 from PVA hydrogel was systematically investigated by considering the effect of the PVA processing parameters. A decrease in release rate was observed when the number of freeze-thaw cycles was increased, the PVA solution concentration was increased, or the freezing and thawing rates were decreased. By manipulating the different processing parameters studied, the diffusive properties of Serp-1 from PVA hydrogel could be controlled over a 30-fold range. In contrast to the

PVA/BSA system where virtually all the protein is released, the amount of Serp-1 released from an equivalent system was found to be only approximately 50 % with the remaining protein trapped in the PVA matrix. This is likely due to a combination of factors including the possible stronger interaction of Serp-1 and PVA, as well as shrinkage of the hydrogel structure.

A therapeutic level of Serp-1 release was achieved by increasing the initial protein loading and with only two freeze-thaw cycles the release was extended over a period of approximately 100 hours. A promising PVA/Serp-1 controlled drug delivery system with tunable properties has been demonstrated. The use of such a controlled release system would offer an improvement over the current Serp-1 delivery method of infusion by gradually releasing Serp-1 over time at the local site of arterial injury. The system can be tuned to adjust the release rate of Serp-1 one by selecting the appropriate combination of processing conditions. The release kinetics can also be adjusted by selecting a one-layer matrix-type system or two-layer reservoir-type system. Therefore, there are many options to tune the PVA/Serp-1 system to meet therapeutic requirements once they are known following clinical trials of the new therapeutic agent.

## **KEYWORDS**

Hydrogel, poly(vinyl alcohol), bovine serum albumin, Serp-1, controlled release, diffusion, freeze-thaw cycles, physical crosslinking

## ACKNOWLEDGEMENTS

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This project would not have been possible without the technical assistance from Viron Therapeutics, Inc. I would like to especially thank Elaine King for her support and input on the Serp-1 work; Andrew Peters for training me to do ELISA; Yunming Sun for assisting with the Western blots; and Dr. Colin Mcaulay and Dr. Xing Li for their discussions and advice. Viron Therapeutics, Inc. also kindly donated all the Serp-1 protein and associated antibodies for this work.

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## LIST OF ABBREVIATIONS

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BMS	Bare Metal Stent
BSA	Bovine Serum Albumin
CABG	Coronary Artery Bypass Graft Surgery
CSLM	Confocal Scanning Laser Microscope
DES	Drug-Eluting Stent
DNA	Deoxyribonucleic Acid
EC	Endothelial Cell
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administration
FITC-BSA	Fluorescein Isothiocyanate Conjugated Bovine Serum Albumin
HPMC	Hydroxy Propyl Methyl Cellulose
HRP	Horseradish Peroxidase
LbL	Layer-by-Layer
K <sub>2</sub> EDTA	Ethylenediaminetetracetic Acid Dipotassium Salt
MMP	Matrix Metalloproteinase
mTOR	Mammalian Target of Rapamycin
M <sub>w</sub>	Number Average Molecular Weight
PAI	Plasminogen Activator Inhibitor
PBMA	Poly(n-butyl methacrylate)
PC	Phosphorylcholine
PCI	Percutaneous Coronary Intervention
PCL	Poly(caprolactone)
PDLLA	Poly(D,L-lactic acid)
PEG	Poly(ethylene glycol)
PEVA	Poly(ethylene-co-vinyl acetate)
PLGA	Poly(lactic-co-glycolic acid)
PLLA	Poly(L-lactic acid)
PVA	Poly(vinyl alcohol)
RSL	Reactive Site Loop
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SIBS	Poly(styrene-isobutylene-styrene)
SMC	Smooth Muscle Cell
TMB	Tetramethylbenzidine
T <sub>g</sub>	Glass Transition Temperature
tPA	Tissue Plasminogen Activator
uPA	Urokinase Plasminogen Activator
uPAR	Urokinase Plasminogen Activator Receptor
UV-Vis	Ultraviolet-Visible

## Chapter 1

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# INTRODUCTION

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### 1.1 BACKGROUND AND MOTIVATION

Cardiovascular disease is a leading cause of death in the Westernized world. Coronary artery disease is most common type of cardiovascular disease and is caused by atherosclerosis. The build-up of plaque on artery walls, or atherosclerosis, results in narrowed or blocked arteries. Atherosclerosis of the coronary arteries is a risk for heart attack and treatment options are crucial. Treatment may include coronary artery bypass graft surgery (CABG) or percutaneous coronary intervention (PCI). PCI has its advantages since it is a much less invasive procedure and can provide almost immediate improvement of the quality of life. PCI involves balloon angioplasty and deployment of a stent to push back the plaque and provide a mechanical structure to keep the vessel open while it heals. The main disadvantage of PCI is restenosis, where re-narrowing within the stented region occurs in the first three weeks following injury, affecting 15-20% of cases [1]. In-stent restenosis is a reaction to the injury caused by the angioplasty balloon expansion and stent implantation, and in addition, may be a foreign body response to the implanted stent [2]. This biological response manifests after endothelial denudation leading to recruitment of inflammatory cells and cellular proliferation and migration of smooth muscle cells (SMCs) and fibroblasts ending in neointimal formation [3-5].

Drug-eluting stents (DESs) have become an important treatment option as they are intended to prevent in-stent restenosis by delivering therapeutics to control neointimal formation. They offer local drug delivery to manage the tissue in-growth leading to

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neointimal hyperplasia. The most common drugs delivered are anti-proliferative agents that have the downfall of not being specific to cell type [6]. Both endothelial cells (ECs) and SMCs are inhibited when re-endothelialization is an important step in maintaining stent patency. Current DESs have problems with late-stent thrombosis, local toxicity, and hypersensitivity reactions [7-10]. There is a need to create new coatings for DESs including the use of new materials and new drugs. The design of such a system would require materials that are biocompatible and hemocompatible as well as having the ability to control the release of a specific drug in a timely manner. The controlled release mechanism and drug-material interaction plays a large role in how this is achieved.

Poly(vinyl alcohol) (PVA) is a common biomaterial and the physically crosslinked class of PVA hydrogels by the low temperature thermal cycling method have been studied for drug delivery applications. PVA is physically crosslinked by repeated freezing and thawing cycles making it more biomedical friendly due to the lack of chemical crosslinking agents. This class of PVA hydrogels have tuneable mechanical properties and controlled release rates based on the processing parameters utilized [11, 12]. The use of PVA as a controlled release matrix has been studied for protein release [13-15]. PVA is an attractive alternative for local drug release in the coronary artery because its hydrophilic nature allows for the delivery of large water-soluble drugs such as protein. The existing polymer coatings on DESs are mostly hydrophobic and are unable to incorporate protein drugs neglecting an important class of drugs which could potentially be useful to control restenosis.

Serp-1 is a serine proteinase inhibitor that is isolated from the myxoma virus. This viral protein has demonstrated anti-inflammatory activity and the ability to reduce plaque growth in animal models following angioplasty injury [16, 17]. Serp-1 is a regulator of the thrombolytic, thrombotic and inflammatory pathways. By targeting particular components of these pathways, Serp-1 has the potential to regulate restenosis by an alternative method as opposed to arresting the cell cycle non-specifically, as done by the popular anti-proliferative agents used on DES platforms.

## 1.2 OBJECTIVES

The overall goal of this thesis project was to design a PVA hydrogel controlled release system to deliver the Serp-1 protein for the management of restenosis.

The objectives are:

- (1) To examine the diffusion properties of PVA hydrogel using bovine serum albumin (BSA) as a model protein
- (2) To observe the difference in release kinetics of BSA from single layered (matrix) PVA hydrogel versus multi-layered (reservoir) PVA hydrogel
- (3) To determine the effect of release medium on the release profile of BSA from PVA
- (4) To characterize the diffusion of Serp-1 from PVA hydrogel
- (5) To model the release kinetics of Serp-1 from PVA hydrogel

## Chapter 2

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# LITERATURE REVIEW

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Percutaneous coronary intervention (PCI), also known as angioplasty, often involves threading a catheter into an artery to the area narrowed by plaque then dilating a balloon to push back the plaque and open the vessel. A metallic stent can be expanded to hold the vessel open after balloon angioplasty. The action of a balloon and stent forcing the artery open leads to injury of the vessel wall. The response to this injury from the overstretching of the arterial wall can lead to restenosis [2, 18]. The re-narrowing of the vessel, or restenosis, within the stented region is the main reason for stent failure and is defined as more than 50 % loss of lumen diameter [19]. Neointimal hyperplasia is the main culprit of in-stent restenosis and is a complex response that starts with disruption of the endothelial layer. This involves platelet activation and the influx of inflammatory cells, followed by cellular migration and proliferation in the intimal layer. Vascular SMCs and fibroblasts travel from the media and adventitia into the intima and begin to deposit extracellular matrix (ECM), remodeling the vessel wall and forming scar tissue that may obstruct blood flow [1, 4, 5].

To prevent restenosis, efforts have been placed on therapeutic treatment. Systemic administration of anti-restenotic drugs has not been successful owing to deficient drug concentration reaching the local arterial target [8, 20]. To alleviate this, local drug administration is used to ensure a high enough drug concentration at the target site is achieved and to minimize adverse systemic effects. Drug-eluting stents (DESs) have been the most successful local delivery method thus far. A DES is a bare metal stent (BMS) coated with an anti-restenotic drug to be released. Typically the drug is in a polymer matrix so that the polymer provides some control over the drug release

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properties. The first and second generations of DESs include four key stents which have been US Food and Drug Administration (FDA) approved: Cypher (2003) by Cordis Corporation, Taxus (2004) by Boston Scientific, and then Endeavor (2008) by Medtronic, and Xience (2008) by Abbott. There are still concerns over the use of the current DESs which may experience insufficient drug delivery [21], potency and non-specificity of drugs [6], delayed healing and late stent thrombosis [7, 22], and local hypersensitivity [9, 10]. Thus, there is a need for a new approach for local drug delivery to fight restenosis, which may include new drugs, new delivery platforms, and/or new devices.

## **2.1 MOLECULES FOR RESTENOSIS REDUCTION**

Following PCI there are multiple molecules which have been considered for restenosis reduction. Therapeutic strategies for preventing restenosis usually target the healing response that leads to neointimal hyperplasia by hindering platelet activation, acute inflammation, SMC migration and proliferation, ECM production, angiogenesis and vascular remodeling [18]. These agents may include anti-platelet and anti-coagulant, anti-inflammatory and immunosuppressant, anti-proliferative, and pro-healing drugs. Local delivery of these anti-restenotic agents has been directed at several biological targets in clinical studies. For example, anti-coagulant molecules such as heparin and argatroban [23] are thrombin inhibitors. Nitric oxide [24] and angiopeptin [25] have been considered for their modulation of SMC proliferation. Several other agents used target the cell cycle at different stages causing cell cycle arrest to slow or stop cell proliferation. These include paclitaxel [26-30], 7-Hexanoyltaxol [31-33], sirolimus [34-36], tacrolimus [37], everolimus [38, 39], zotarolimus [40, 41]. Actinomycin D [42] inhibits RNA synthesis, while batimastat [43] inhibits matrix metalloproteinases (MMPs). Dexamethasone [44-46] and mycophenolic acid [47] are both immunosuppressants. Pro-healing agents VEGF genes [48], estradiol [49, 50], and anti-CD34 antibodies [51] all promote endothelialization.

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## 2.2 DRUGS FOR DRUG-ELUTING STENTS

Delivery of drugs from DESs must take into consideration factors such as the therapeutic concentration range, timeframe of delivery, and local pharmacokinetics. Local delivery of these drugs to the injured site allows for prolonged delivery at high concentrations without the risk of systemic toxicity and avoids the loss of agents with short half lives [8]. Yet, it is still essential that the concentration of the drug be within the therapeutic range so that it is high enough to be effective but not toxic [21]. The timing of drug release should match the healing of the arterial wall and it is thought a timeframe of around 30 days for most anti-restenotic drugs is required [52, 53].

Heparin, a thrombin inhibitor, has been the common anti-coagulant studied for DESs due to its extensive clinical use [54], but other agents that prevent the cascade of events leading to coagulation, such as anti-platelet drugs, are also thought to be useful but have been met with limited success [4]. Dexamethasone is of interest because it is known for its anti-inflammatory properties [44]. Pro-healing agents provide a different approach by promoting re-endothelialization so the arterial wall heals quickly and restores normal function to preserve stent patency. The endothelial wall is critical as it provides anti-thrombotic factors and without complete re-endothelialization late stent thrombosis is a risk [6, 7]. The most popular therapeutic strategy is the use of anti-proliferative agents. Commercially used DESs in America all have anti-proliferative agents on their platforms. These include paclitaxel and sirolimus as well as some derivatives of sirolimus, which will be discussed in the following sections.

### 2.2.1 Paclitaxel

Paclitaxel is an anti-proliferative agent as a result of its ability to stabilize microtubules causing cell cycle arrest at the mitotic phase, rendering the microtubules dysfunctional and incapable of cell replication [55-57]. Also known by its trade name Taxol, it is commonly used for cancer treatment for its ability to cause cell death in



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replicating cancer cells. Paclitaxel is found in the extract from the bark of Pacific Yew tree [55].

Paclitaxel is the active ingredient of the Taxus Express stent by Boston Scientific. Clinically, this DES has shown that it is more effective in reducing restenosis when compared to a BMS as demonstrated in the TAXUS I-IV trials [26-29, 58-60].

### **2.2.2 Sirolimus and its Derivatives**

Sirolimus (rapamycin) is also known for its anti-proliferative properties. Its mode of action differs from paclitaxel in that it inhibits the mammalian target of rapamycin (mTOR), arresting the cell cycle at the transition of G1 to S phase [61]. It is also thought to have immunosuppressant properties potentially making a more attractive option. Its immunosuppressant activity comes from the propensity to inhibit cell cycle progression in T lymphocytes [62]. Sirolimus is a fermentation product of a soil microorganism from Rapu Nui [61].

Several sirolimus-analogues have also been considered for their potential use as anti-restenotic drugs. For instance, everolimus has a similar mode of action and is the active agent of Abbott's Xience V stent [63]. Zotarolimus, used on Medtronic's Endeavor stent, is another analogue that has a shorter circulating half-life, reducing its systemic immunosuppressant potency [64].

## **2.3 SERP-1**

A new therapeutic agent still under clinical trials is Serp-1 for its potential anti-inflammatory and anti-atherogenic properties. Serp-1 is a serine proteinase inhibitor (serpin) that is secreted by the myxoma virus, a pathogen of rabbits. It is a 55 kDa glycoprotein with promise as an anti-inflammatory agent as it has been shown to modulate immune cell responses [65].

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A phase I safety trial in man of Serp-1 was successful and no adverse side effects were observed. Currently, it is undergoing a phase IIa safety and efficacy trial where patients are given an infusion of Serp-1 immediately after balloon angioplasty and stent implantation. It is the first human trial of a native anti-inflammatory viral protein [66].

### **2.3.1 Biological Role and Therapeutic Effect**

The activity of Serp-1, like other serpins, is dependent on its conformation and reactive site loop (RSL). To maintain their inhibitory activity, serpins stay in an unstable conformation with the RSL sticking out of the  $\beta$ -sheet. The more stable state with the RSL inserted in the  $\beta$ -sheet gives a non-inhibitory or latent state similar to a cleaved serpin [67]. They operate via a suicide inhibition, where the RSL binds to a protease and drags it across the face of the serpin changing the native conformation of both the serpin and protease, rendering both inactive. Alteration of the RSL of Serp-1 results in loss of protease inhibitory activity and anti-inflammatory activity [68, 69].

Serpins are known for the inhibitory activity acting as regulators of many proteolytic cascades [67]. As a serpin, Serp-1 is considered for its regulation of thrombosis, thrombolysis, and inflammatory pathways. The thrombotic cascade results in clotting from both intrinsic and extrinsic cascades leading to activation of factor X and thrombin formation. Thrombin activates fibrinogen to form fibrin, creating a clot. The thrombolytic cascade is responsible for clot dissolving. Thrombolytic participants tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) cleave the pro-form of plasminogen to form active plasmin. Playing a part in inflammation, tPA, uPA, uPA receptor (uPAR) and their serpin, plasminogen activator inhibitor (PAI-1), are up-regulated at sites of tissue injury. The plasminogen activators along with plasmin activate MMPs to degrade connective tissue, collagen, and elastin, allowing invading cells to migrate in damaged tissue. The plasminogen activators also release growth factors. Excessive uPA or PAI-1 has been associated with increased plaque growth and increased inflammation [70, 71].

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Serp-1 is a native viral immunomodulatory protein that redirects the host immune response away from the invading virus. It is an inhibitor of tPA, uPA and plasmin from the thrombolytic pathway and an inhibitor of factor Xa from the thrombotic pathway [72]. Thus, Serp-1 has the potential of being a strong regulator of the cascades directly affecting arterial plaque and inflammation. In the presence of heparin, Serp-1 inhibits thrombin and has the potential to be more anti-thrombotic than anti-thrombolytic [73]. Serp-1 also has been shown to block activation of ECs, platelets, monocytes, and T cells which signifies its ability to regulate immune responses [74, 75].

### **2.3.2 Animal Studies**

Purified Serp-1 has been used in animal models to demonstrate its capability to diminish inflammation and cell migration. For instance, in a rabbit model using angioplasty injury, one bolus of 30-3000 ng of Serp-1 was infused with a Wolinsky catheter. This low dose, local infusion was given at the time of balloon angioplasty and resulted in effective inhibition of plaque growth at 4 weeks and blocked early inflammatory cell invasion [17].

In other studies of angioplasty injury in rabbit aorta or microswine models, Serp-1 was not effective after a single bolus immediately after stent implant. However, plaque was reduced after 2, 3 or 11 daily intravenous injections after stent implant [16, 66]. Therefore, with recurring injury such as occurs with repeated angioplasty or an implanted stent, there was less effective reduction in plaque with a single bolus, but multiple injections significantly reduced plaque [16, 66]. This study also confirmed that Serp-1 reduced monocyte and T-cell invasion at the site of vascular injury [16].

In another study, a mouse model of carotid cuff injury was evaluated. Serp-1 was infused subcutaneously continuously by an osmotic pump for 4 weeks [76]. It was found there was a significant (67 %) reduction in plaque size and a reduction in markers for plaque instability. However, if treatment was started 5 weeks after cuff placement instead of one week, there was no significant reduction observed [76]. It was also shown

that macrophage number was decreased with increased SMCs and collagen content, signifying a more stable plaque phenotype with less risk of plaque rupture [76].

Serp-1 has also been investigated in models for aortic transplant [77], renal transplant [78], arthritis [79], and angiogenesis [80].

## **2.4 CONTROLLED RELEASE**

Drug delivery can take many forms from patches, to injected microspheres, to pumps, to tablets, to implants, and so on. Any drug delivery device has the goal of delivering a therapeutic agent to a target site. Improving upon this is the concept of controlled release. Controlled release provides a method to deliver the drug for a specific and predictable rate, dose and timeframe. While simply getting the drug to a targeted local site is enough for some applications, quite often it is important to control the release kinetics so that the drug is effective for an appropriate amount of time. This includes maintaining the drug concentration within a therapeutic range so that it does not become toxic if it is too high or so that it is ineffective if it is too low. Polymeric systems are the dominant approach for controlled release systems, but non-polymer based systems are also available including liposomes, porous metallic surfaces and pumps [81-83]. However, the discussion of this thesis will be restricted to polymer based systems as it directly relates to the current study.

### **2.4.1 Release Mechanisms**

Several release mechanisms exist for controlling drug delivery from polymeric systems, such as diffusion, degradation, ion-exchange, osmosis, and chemically modified. For drug delivery, physical mechanisms are more easily achieved than chemical mechanisms. Chemical mechanisms have the disadvantage of having to chemically modify the drug to tether it to the delivery polymer [21]. Therefore, physical mechanisms are easier to use for controlled drug delivery and can be quite effective. The

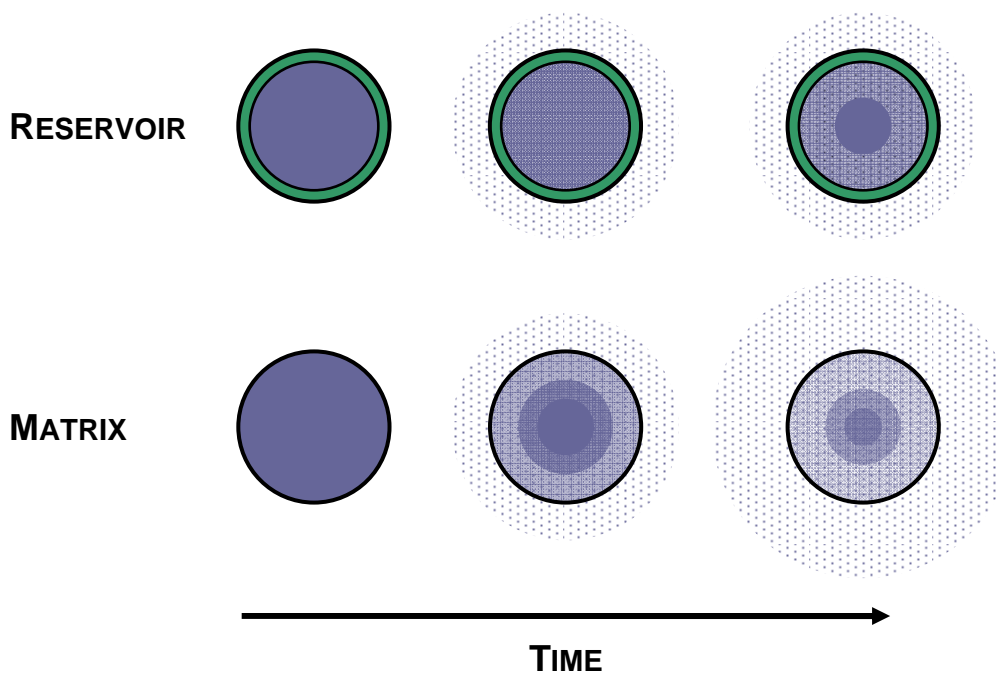
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mechanism of release predicts the release kinetics which can be modified by varying the geometry of device, the thickness of polymer membrane, surface area, type of polymer used, and so on [21]. In fact, the controlled release properties of a specific system are dependent on several factors, mostly to do with the properties of the polymer, the properties of the drug, and their interaction. Properties of the polymer to be considered include molecular weight, degree of crosslinking, hydrophobicity, etc. Important properties of the drug may include its solubility in the polymer, size, surface charge, etc.

Diffusion controlled release simply involves embedding a drug into a polymeric matrix and allowing it to migrate out of the matrix based on a concentration gradient of the drug between the surrounding environment and inside the polymer matrix. The rate at which the drug diffuses is dependent the distance it has to travel and the tortuosity of the path which is based on the properties of the polymer. The kinetics of this mechanism will be further discussed in the following section. Degradation controlled release is dependent on the degradation or dissolution of the polymer with drug encapsulated, releasing the drug as the polymer breaks down. When the time period of degradation is much greater than the release period, the degradation rate of the polymer controls the rate of release [84]. Ion exchange release means that the drugs are electrostatically bound to the polymer matrix and can be exchanged with ions of the same charge from the surrounding environment. Ion exchange is a promising approach for deoxyribonucleic acid (DNA) delivery due to it being a charged molecule [21]. Osmotically controlled release typically involves swelling an osmogen or salt-containing device that puts pressure on a drug depot, forcing the drug out through an orifice. The release kinetics are zero-order because of the constant osmotic pressure that builds as water is drawn across a semi-permeable membrane to the osmogen, and swelling squeezes the drug out at a steady-state [85]. Swelling controlled release involves water moving into the polymer network and swelling the polymer matrix so that there are larger pores and more space for the drug to diffuse out. The macromolecular chains relax as the polymer transforms from the glassy state to a rubbery state allowing diffusion to occur [86].

### 2.4.1.1 Diffusion Controlled Release: Matrix versus Reservoir Systems

There are two systems for release by diffusion, matrix-type or reservoir-type. A matrix-type device contains a drug that is uniformly distributed throughout a polymer matrix. A reservoir-type device has a drug core which is surrounded by an outer layer or membrane. FIGURE 2.1 demonstrates the differences in the two systems.



**FIGURE 2.1.** Reservoir-type and matrix-type drug delivery systems. Reservoir-type contains a saturated drug reservoir surrounded by a rate-controlling membrane of constant thickness and diffusivity. Matrix-type has drug uniformly dispersed in a polymer matrix and drug depletes over time.

A reservoir drug delivery system should display release that is independent of time with zero-order kinetics. The release rate is controlled by diffusion through the outer membrane [87]. The thickness of the membrane can be adjusted to regulate the release rate, making it easy to alter the dosage without changing the formulation. The drug reservoir can contain the concentrated drug alone or can be a polymer matrix within which the drug is suspended. In the case of the latter, the polymer matrix should provide

less diffusional resistance than the outer rate-limiting membrane layer. The release kinetics for this type of system follow Fick's first law, under sink conditions, where the concentration of the drug core is sufficiently high so that the concentration difference on either side of the membrane is constant. This constant flux ( $J$ ) is described by EQUATION 2.1, where  $D$  is the diffusion coefficient,  $C$  is the drug concentration,  $\frac{\partial C}{\partial x}$  is the concentration gradient along position  $x$ ,  $K$  is a partition coefficient, and  $l$  is the thickness of the membrane [88].

$$J = -D \frac{\partial C}{\partial x} \quad \text{or} \quad J = \frac{DK\Delta C}{l} \quad (\text{EQUATION 2.1})$$

A disadvantage of the reservoir-type drug delivery system is that a sudden rupture of the outer membrane would lead to the release of the drug all at once or 'dose dumping'. This has the obvious problem of resulting in sudden local toxicity. Another disadvantage of this type of system may be the occurrence of a time-lag or burst effect. A time-lag occurs when the drug molecule takes some time to cross the outer membrane barrier before releasing at a steady state. The opposite can occur resulting in a burst which may happen if the drug has migrated into the membrane layer during storage and rapidly releases at the beginning before steady state is reached [89].

Matrix-type (monolithic) drug delivery systems are polymeric matrices with drug molecules dissolved or dispersed. The release rate is time dependent and influenced by the initial drug loading and geometry [87]. Drug release occurs by diffusion of the drug to the surface and is controlled by the properties of the polymer matrix. The main disadvantage of matrix-type systems is that the release is not zero-order.

For drugs that are soluble within the matrix there are two cases depending if the drug concentration is above or below the concentration of its solubility within the matrix. When the drug concentration is less than its solubility in the matrix, the release rate decreases over time as molecules near the surface have a shorter distance to travel and release first [84]. In fact, for slab geometry the cumulative release is proportional to the

square root of time. This process is described by Fick's second law (EQUATION 2.2) since the concentration of the drug within the volume is changing with time  $\left(\frac{\partial C}{\partial t}\right)$ .

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} \quad (\text{EQUATION 2.2})$$

When the drug concentration is greater than its solubility in the matrix, the release rate is limited by the drug dissolution. Drug rich phases must first dissolve into the polymer matrix before diffusing to the surface. In this instance, the famous Higuchi model (EQUATION 2.3) can be used to describe the pseudo steady state kinetics [90]. Here  $M_t$  is the amount of drug released,  $A$  is the surface area,  $C_s$  is the drug solubility in the matrix, and  $C_o$  is the initial drug concentration. Assumptions made for this equation are that  $C_o \gg C_s$ , swelling is negligible, perfect sink conditions are maintained, and edge effects are negligible [86, 91].

$$M_t = A[DC_s(2C_o - C_s t)]^{1/2} \quad (\text{EQUATION 2.3})$$

For drugs that are not soluble in the polymer matrix, diffusion must occur through water-filled pores created by the drug particles themselves. An example of such a system would be water-soluble protein in a hydrophobic polymer. In this case, the solid drug particles are dispersed throughout the polymer and dissolve when placed in contact with an aqueous environment. The drug molecules do not diffuse through the polymer phase but through the water-filled network created as the drug particles dissolve [84]. This requires interconnecting pores be created by the solid drug particles. Therefore, the drug loadings need to be high since low loadings yield disconnected pores. Often with these systems the release is not 100 % since there is always the possibility of isolated particles. Larger drug particles and higher loadings result in increased release rates due to the simpler pathways they create. For these systems an effective diffusion coefficient ( $D_{eff}$ ) can be found (EQUATION 2.4) based on the tortuosity ( $\tau$ ), porosity ( $\varepsilon$ ), and the diffusion coefficient in the water-filled pores ( $D_{pore}$ ) [84].



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$$D_{eff} = \frac{D_{pore} \cdot \varepsilon}{\tau} \quad (\text{EQUATION 2.4})$$

## 2.4.2 Empirical Models

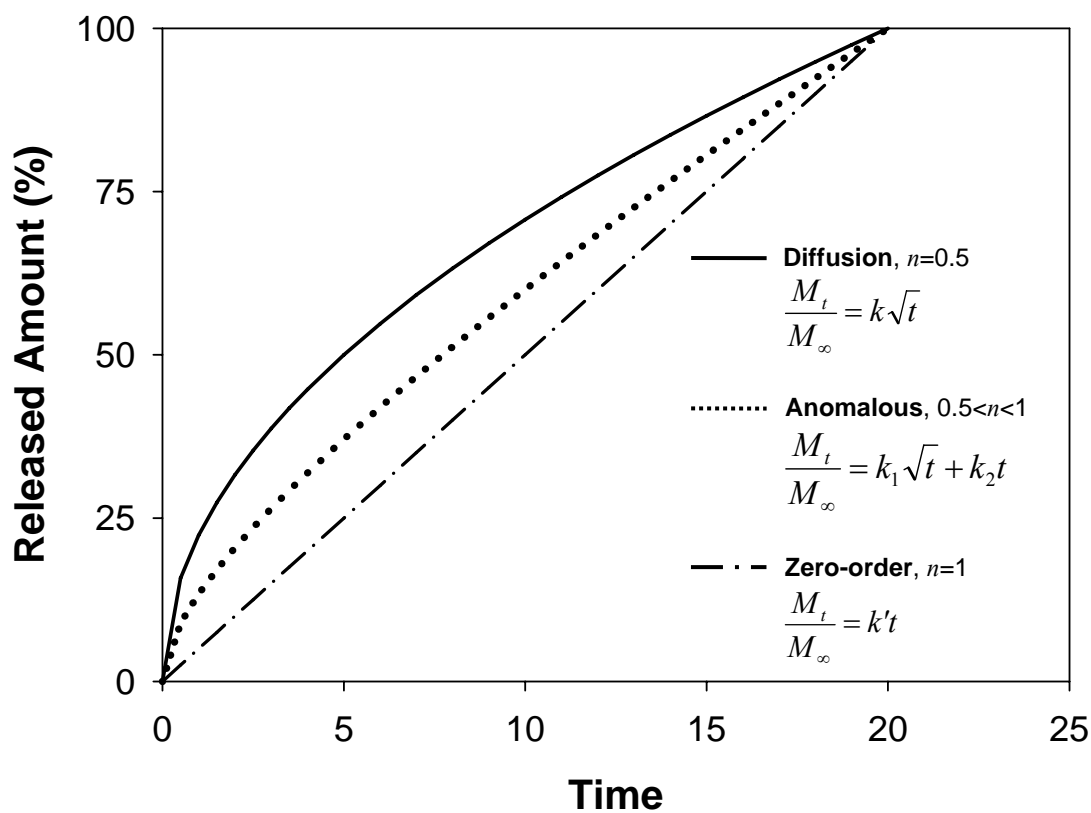
Mathematical modeling of controlled drug delivery systems provides information on release kinetics and transport processes. Diffusion is the most frequently modeled process as it is the common mechanism for pharmaceutical controlled release applications. Empirical or semi-empirical models help to describe the movement of molecules through the polymeric matrix.

### 2.4.2.1 Power Law

The Power Law was first described by Ritger and Peppas in 1987 for drug release purposes [92]. This semi-empirical model (EQUATION 2.5) is used to analyze the behaviour of drug release and is valid for the first 60 % of release. Here,  $M_t/M_\infty$  is the fraction of drug released, with  $M_t$  being the amount of drug released at time  $t$  and  $M_\infty$  the infinite drug loading,  $k$  is a kinetic constant and  $n$  is the release exponent [92].

$$\frac{M_t}{M_\infty} = kt^n \quad (\text{EQUATION 2.5})$$

The value of the release exponent  $n$  provides useful information on the mechanism of release. FIGURE 2.2 demonstrates the differences in release profiles for varying  $n$  values. For thin slab geometry, an  $n$  value of 0.5 indicates Fickian diffusion [92]. For other geometries and release mechanisms, the value of  $n$  can be found in TABLE 2.1.



**FIGURE 2.2.** Release profiles of different release mechanisms, demonstrating the significance of the release exponent.

**TABLE 2.1.** Release exponent,  $n$ , for different release mechanisms and geometries [92].

Release Exponent, $n$			Drug Release Mechanism
Thin film	Cylinder	Sphere	
0.50	0.45	0.43	Fickian diffusion
$0.50 < n < 1.0$	$0.45 < n < 1.0$	$0.43 < n < 1.0$	Anomalous
1.0	1.0	1.0	Zero-order release

### 2.4.2.2 Diffusion Model

The Diffusion Model arises from Fick's second law of diffusion under the assumption of one-dimensional release under perfect sink conditions. Specifically for thin slab geometry, the solution can be represented as a series (EQUATION 2.6) or an error function (EQUATION 2.7) [88].

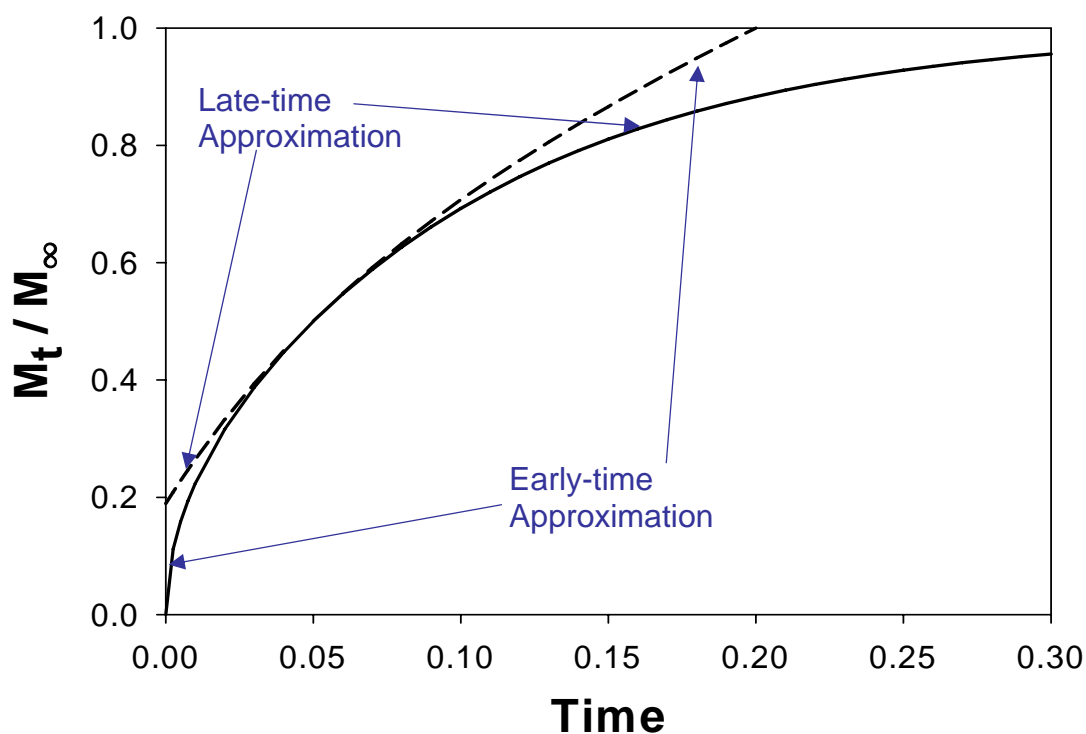
$$\frac{M_t}{M_\infty} = 1 - \sum_{n=0}^{\infty} \frac{8 \exp(-D[2n+1]^2 \pi^2 t / l^2)}{(2n+1)^2 \pi^2} \quad (\text{EQUATION 2.6})$$

$$\frac{M_t}{M_\infty} = 4 \left( \frac{Dt}{l^2} \right)^{1/2} \left( \pi^{-1/2} + 2 \sum_{n=0}^{\infty} (-1)^n \operatorname{ierfc} \frac{nl}{2\sqrt{Dt}} \right) \quad (\text{EQUATION 2.7})$$

These complex equations can be simplified for everyday use into a short-time approximation (EQUATION 2.8) and a long-time approximation (EQUATION 2.9) [88]. A plot of the two approximations is shown in FIGURE 2.3.

$$\frac{M_t}{M_\infty} = 4 \left( \frac{Dt}{\pi l^2} \right)^{1/2} \quad \text{for } 0 \leq \frac{M_t}{M_\infty} \leq 0.6 \quad (\text{EQUATION 2.8})$$

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2} \exp\left(-\frac{\pi^2 Dt}{l^2}\right) \quad \text{for } 0.4 \leq \frac{M_t}{M_\infty} \leq 1.0 \quad (\text{EQUATION 2.9})$$



**FIGURE 2.3.** Early-time and late-time approximations of the Diffusion Model. Adapted from [88].

### 2.4.3 Delivery of Large Water Soluble Drugs (Protein)

Although polymer matrices make for good delivery vehicles for drugs, the commonly used polymers are hydrophobic and best accommodate hydrophobic drug molecules. However, this eliminates the use of a range of hydrophilic drugs of therapeutic benefit. In particular, proteins are a large class of therapeutics which are typically water-soluble and sensitive agents that can be challenging to deliver effectively.

The biological activity of a protein can be difficult to maintain until reaching its target. Oral delivery of proteins is limited by the gastric system and thus multiple injections or intravenous infusions become the delivery methods of choice [93]. Local drug delivery of protein therapeutics would overcome the difficulties of patient compliance and also help to retain the protein activity, increasing its efficacy.

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Incorporating a protein drug into a polymer matrix can protect the protein from proteolysis and antibody neutralization *in vivo* in addition to providing a controlled release vehicle [94]. Design of a controlled release system for a protein drug should take into consideration properties of the protein including molecular size, biological half-life, immunogenicity, conformational stability, dose requirement, site and rate of administration, pharmacokinetics, and pharmacodynamics [93, 95].

It can be a challenge to incorporate proteins into a polymer matrix. Complications that may be encountered during manufacturing include protein denaturing by chemicals used, leaching out of the protein in aqueous solutions while removing porogens from the polymer matrix, and loss of protein activity when chemically tethering to the polymer backbone [96]. To avoid these problems, hydrogels with high water content are viewed as protein friendly delivery materials. Water-soluble proteins can easily diffuse through a hydrogel matrix with only its size as a restriction [96]. Swollen hydrogels offer more effective area for diffusion of larger macromolecular drugs [93]. The downfall of using hydrogels for controlled release applications is that the release rate is often rapid, but there are tactics to alleviate this. For instance, the crosslinking density can be increased to decrease the diffusion of the protein [96].

## **2.5 MATERIALS FOR DRUG-ELUTING STENT COATINGS**

Polymers are thus far the material of choice for DES coatings. The coating of a DES has several roles including being a surface that is friendly in the blood-contacting cardiovascular environment as well as being able to control the release of the drug it contains. These devices are implanted in direct contact with the bloodstream, therefore they must not only be biocompatible, but also preferably be hemocompatible. Hemocompatibility means that the material properties will not change blood functions, transform blood components or form thromboses [97]. The interaction between blood and a foreign material can initiate protein adsorption, platelet adhesion and activation, leukocyte adhesion and activation, and the activation of the complement and coagulation

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pathways [98]. However, in general all synthetic polymers are blood incompatible, thus the challenge becomes to use materials and techniques to increase their blood compatibility. To aid in hemocompatibility, materials with a surface that is non-thrombogenic are crucial. The surface properties of a material greatly influence its thrombogenicity. These include surface energy, wettability, texture, and charge [53, 99]. Hydrophobic surfaces tend to absorb more plasma proteins than uncharged hydrophilic surfaces. And, smooth surfaces are less likely to adsorb protein while rough surfaces can be strong activators of blood platelets.

Several approaches have been undertaken to modify a material's surface to enhance its hemocompatibility. Three approaches to this are surface passivation to minimize blood-material interaction, immobilization of bioactive molecules on the surface, and promotion of endothelialization [100]. Passive coatings can be biologically inert materials that act as a barrier between stent and the bloodstream, including gold, carbon, and silicon carbide [19, 53]. Heparin, an anti-coagulant, is a frequently employed molecule that can be immobilized on the surface by endpoint attachment for improved hemocompatibility [54]. Additionally, the promotion of endothelialization of a surface has gained some attention in recent years as a method to create a hemocompatible surface that mimics nature. This can be achieved by two different approaches. The first approach is to seed a material's surface with ECs with the help of cell adhesion proteins or peptide sequences, allowing a confluent layer to cover the surface before implantation. The second approach is to either immobilize a molecule to the surface or release molecules that attract ECs from the bloodstream *in vivo*. This includes antibodies that capture endothelial precursor cells to encourage endothelialization [51].

In addition to surface properties, some other important criteria exist for the materials used to coat stents. This encompasses the stent and coating stability after placement, the solubility compatibility between polymer, solvent and drug during coating process, the coating stability, expandability and integrity during stent deployment, and the materials must be sterilizable [101, 102].

Another major design criterion for the DES coating material is its ability to provide controlled drug delivery. These devices contain an active ingredient to help reduce restenosis. Therefore, the polymer coating must allow the drug to be delivered in a timely method, ensuring consistent dosing and release kinetics that are controlled and predictable [102]. Later sections of this thesis will discuss controlled release factors in further detail.

The first DESs are coated with hydrophobic elastomers. However, Medtronic's Endeavor stent takes a new approach using phosphorylcholine (PC) as a coating material and Abbott's Xience V stent uses a fluorinated copolymer. Biodegradable coatings and materials for fully degradable stents are currently being researched intensely.

### **2.5.1 Elastomers**

Elastomers were originally the material of choice for stent coatings for their ease of incorporating the common hydrophobic drugs and expandability for stent deployment. The Cypher is coated with a combination of poly(ethylene-co-vinyl acetate) (PEVA) and poly(n-butyl methacrylate) (PBMA). The Taxus is coated with poly(styrene-isobutylene-styrene) (SIBS). These elastomers have disadvantages including lack of control over release without creating a new formulation, where drug release is essentially modified by the drug loading and/or the addition of polymer layers and not the properties of the material itself. A further disadvantage may be hypersensitivity as reactions have been reported [9, 10, 103].

### **2.5.2 Phosphorylcholine**

PC is a more recently investigated material for stent coatings since it mimics the cell membrane by containing both hydrophilic and hydrophobic components. The outer edge of the coating is hydrophilic for a reduced thrombogenic potential whereas the inner layer is lipophilic and acts as drug reservoir [19, 104]. Clinically, PC is used on

Medtronic's Endeavour stent and Biompatible's BiodivYsio stent. On the BiodivYsio DES, a range of drugs have been applied and are loaded onto the stent by immersing the stent in an alcoholic or aqueous solution of the drug [105]. Water-soluble drugs release more rapidly than hydrophobic drugs which are delayed by the hydrophobic drugs interacting with the hydrophobic domains of the PC [105]. Dexamethasone, an anti-inflammatory, is absorbed on the BiodivYsio platform and marketed in Europe by Abbott as the Dexmet stent [44-46]. Other drugs that have been investigated with PC coatings include angiopeptin [25, 105], estradiol [50], batimastat [43], c-myc antisense oligonucleotide [104] and therapeutic genes [106-108]. Compared to other polymer coatings on DESs, PC coated stents have demonstrated less thrombus formation, decreased inflammatory response, and less adverse effects on the rate of re-endothelialization [109-112].

### **2.5.3 Bioresorbable Materials**

Biodegradable or bioresorbable materials have been investigated for two different approaches to DESs. As a coating that disintegrates over time leaving behind a metal stent, or a fully degradable/resorbable stent that props open the blood vessel until it has finished healing and remodeling. Either can be used for drug delivery, but a full polymeric stent provides more volume for drug loading than a coating. There are advantages and disadvantages of fully biodegradable stents. While they avoid a permanent metallic implant, leave access for future revascularization procedures, and reduce late stent thrombosis, they are limited by early recoil and possible severe inflammatory reactions from polymer degradation products [113].

Drug release from degradable polymeric materials can be mostly controlled by the degradation rate and is influenced by viscoelasticity and glass transition temperature ( $T_g$ ), molecular weight, and hydrophilicity. Polyesters are the commonly studied class of degradable materials for this application. Polyesters degrade by hydrolysis with some contribution from enzymatic digestion. The most common polyester, poly(L-lactic acid)



(PLLA), is stiff with high tensile strength and  $T_g$ . Poly(caprolactone) (PCL) degrades slower taking one to two years, due to its high crystallinity and hydrophobicity. Poly(lactic-co-glycolic acid) (PLGA) is amorphous and relatively more hydrophilic allowing for a faster degradation in a few months [114].

An example of a biodegradable stent is Abbott's BVS stent, a PLLA scaffold that elutes everolimus. The PLLA has a poly(D,L-lactic acid) (PDLLA) coating which houses the everolimus and controls the drug release, releasing 80 % of the drug in 28 days. The ABSORB clinical trial demonstrated that at six months the angiographic in-stent late loss was comparable to that of the Taxus and the Endeavor stents, but more than that of the Xience V DES [115]. The late loss was attributed to stent recoil which resulted in a reduction of stent area. The two year follow-up of this trial confirmed the stent was fully bioabsorbed, vasomotion was restored, and no restenosis was observed suggesting late thrombosis was improved [116].

#### **2.5.4 Controlled Release for Drug-Eluting Stents**

DESs are drug delivery devices for which it is important that they also offer control over the drug release. Like many drug delivery applications a prolonged or sustained delivery may be desired depending on the therapeutic released. Additionally, since the drug is delivered locally to the arterial wall, the dose delivered must be controlled within the therapeutic range. The appropriate timeframe and dosage is dependent on the therapeutic agent used as they all have different requirements and biological targets.

Drug delivery should coincide with the vessel wall remodeling and healing [52], and generally 30 days of release is necessary [53]. The therapeutic agents released ideally would inhibit neointimal hyperplasia by suppressing platelet activation, acute inflammation, SMC migration and proliferation, ECM production, angiogenesis and vascular remodeling [18]. For DESs carrying anti-proliferative agents, a general rule of thumb is that the agent must be delivered for at least three weeks since this is the critical

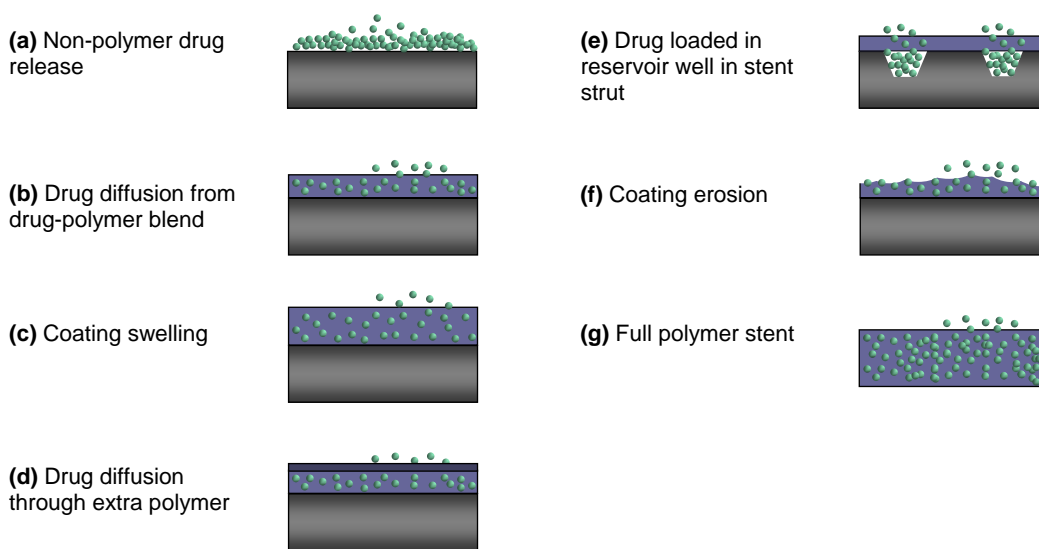
time in which the SMCs are migrating and proliferating [20]. Other timeframes may be required for agents that prevent restenosis by targeting other biological processes.

Local pharmacokinetics are dependent on transport of the drug through the tissue by diffusion and convection as well as the drug properties including molecular weight, charge and hydrophobicity [117, 118]. Hydrophobic drugs take longer to elute and are retained in the arterial tissue, while hydrophilic drugs elute faster and are retained for less time but distribute more evenly in the tissue [52, 119]. Thus it is beneficial to use a hydrophobic drug when high tissue concentrations are preferred, but a hydrophilic drug when there is a narrow therapeutic concentration window [118]. Equilibrium, transport properties and binding capacity affect drug retention. When the threshold of drug adsorption is reached, further drug released will be washed away. Therefore, the drug efficiency is not strictly based on the release kinetics alone and sometimes restenosis may be inhibited with a bolus delivery of drug without sustained release [120]. The response is a function of drug dose, exposure time, drug absorption ability, degree of injury, and the repairing process [121]. However, controlled release plays an important role in more than one of those actions.

For the current DESs, diffusion from a polymer coating is the method of choice. FIGURE 2.4 presents a schematic of possible drug release mechanism for a DES. Some drugs, like paclitaxel [122], can be coated directly on the metallic stent (FIGURE 2.4(a)), but most agents require a polymeric matrix. The polymer coating can also provide enhanced control over drug release. Diffusion from a single layer of polymer coating is a common format for DESs and operates as a matrix-type release device (FIGURE 2.4(b)). Release from a polymer coating can also be achieved by swelling controlled release with a polymer matrix that swells (FIGURE 2.4(c)). Diffusion can also occur via reservoir-type release by adding an additional drug-free polymer layer (FIGURE 2.4(d)). An additional drug release platform for DESs that has been studied is the use of drug reservoir wells [123], where the drug is contained in wells on the stent strut and releases through a polymer coating (FIGURE 2.4(e)). Biodegradable polymers can also be used for DESs, either as a coating on a metallic stent (FIGURE 2.4(f)) or as a fully biodegradable stent

(FIGURE 2.4(g)) and drug release can be controlled by the degradation or erosion of the polymer.

The Cypher stent elutes sirolimus from a combination of PEVA and PBMA. One version of the Cypher is a reservoir-type device with a top coat to prolong the delivery. However, the *in vitro* release profile demonstrates a burst effect before a steady state is reached [21]. Most of the drug is released in three weeks with the remaining extending to 90 days [124]. The Taxus stent elutes paclitaxel from SIBS. For this DES, release is by diffusion but in the case where the drug is in excess of its solubility in the matrix, with the release following the Higuchi model (EQUATION 2.3). Paclitaxel does not dissolve in the polymer but exists as discrete particles embedded in SIBS [102], and its release occurs by dissolution of the paclitaxel into the release medium. For Taxus, the *in vitro* release profile demonstrates a biphasic release pattern by way of a short burst release of 2 days that is dissolution controlled and then a long and slow sustained release of 10 days that is diffusion controlled [102, 125]. The Endeavor releases Zotarolimus from a PC coating where it releases 75 % of the drug in 2 days and 100 % within the first 10 days [126]. The Xience V stent releases everolimus from a fluorinated copolymer and releases 75 % within the first 30 days and 100 % in up to 120 days by a diffusion mechanism [63].



**FIGURE 2.4.** Different possible mechanisms of drug release from a DES platform. Grey represents stent strut, blue is the coating and green is the drug. Adapted from [20].

Much research exists on improving on current drug release strategies for DESs. One example, is the use of the BiodivYsio stent for DNA delivery. The positively charged surface of the PC coating is used to attract the negatively charged DNA molecules. Ion exchange is the release mechanism, where the DNA should be replaced by negative ions in the body such as chloride [21, 127]. Another example is the layer-by-layer (LbL) method typically involving coating the stent in layers of drug and polymer. A multilayered phospholipid polymeric hydrogel was used to incorporate paclitaxel. The hydrophobic domains were used to incorporate paclitaxel into the hydrogel [128]. In another study, polyelectrolyte layers were created with oppositely charged heparin and chitosan. Heparin was meant to reduce thrombus formation and chitosan was to encourage cell attachment and growth in the hopes of promoting rapid healing of the endothelium. [129]

## **2.6 PVA AS A DELIVERY MATRIX**

PVA is a non-toxic, water-soluble polymer. PVA hydrogels can be formed by chemical crosslinking [130-132], UV-irradiation [133], annealing [134], or low temperature cycling [135]. For biomedical applications, physical methods are preferred over chemical methods using toxic crosslinking agents such as glutaraldehyde. Physically crosslinked PVA hydrogels by low temperature thermal cycling, freeze-thaw methods have been extensively investigated [135-141].

The process of creating PVA hydrogels by the freeze-thaw method involves freezing PVA solutions to below the freezing temperature of PVA and thawing back to room temperature repeatedly. The result is a thermoreversible gel produced by a combination of hydrogen bonding, polymer crystallite formation and liquid-liquid phase separation producing a heterogenous structure with phase separated domains [142, 143]. The structure of these hydrogels has been studied by several approaches including transmission electron microscopy [144] and scattering techniques including small angle x-ray scattering [144] and small angle and ultra small angle neutron scattering [145, 146].

The thermal cycling produces a hydrogel with a polymer rich region and polymer poor region [145, 147-150]. The initial freeze-thaw cycle develops ice crystals in the amorphous regions which force the polymer chains into regions of high local polymer concentration [138, 145, 147]. Additional freeze-thaw cycles leads to increasing the size and number of crystallites. The polymer chain-linking crystallites form around 3 nm and are spaced by amorphous regions that span 19 nm [145]. Polymer poor regions are created from the melting of ice crystals during thawing, creating water-filled pores of dimension greater than 100 nm [145, 151].

Several processing parameters affect the structure of PVA hydrogels, including the molecular weight of PVA, the PVA solution concentration, and freezing and thawing conditions [11, 147, 148, 152, 153]. Increasing the number of freeze-thaw cycles increases the mechanical properties [11] and decreases the controlled release rates [12]. The pore size decreases with either an increase in polymer concentration and/or number of freeze-thaw cycles [140, 142]. The size and density of the crystallites decreases with an increased freezing rate [154].

Using the processing parameters, the mechanical properties of PVA hydrogels can be tailored for applications with properties comparable to soft tissues ranging from skin to cardiovascular tissue [11]. Since controlled release properties are closely related to the structural properties, they too should be easily tailored for specific applications.

### **2.6.1 Diffusive Properties of Poly(vinyl alcohol)**

PVA hydrogels by the freeze-thaw method have been investigated for drug delivery and controlled release applications [13, 135, 143, 152, 155-157]. The physical crosslinking method avoids the chemical crosslinking reactions that may deactivate bioactive compounds for delivery. In particular, physically crosslinked PVA hydrogels are non-toxic and hydrophilic making them good for protein delivery matrices.

Protein release from PVA hydrogels occurs by a diffusion controlled mechanism [13, 153]. The structure of the hydrogel plays an important role in the diffusive

properties of the hydrogel as the protein must migrate through the mesh of polymer chains. Diffusion typically occurs very rapidly from hydrogel matrices therefore it is critical to have methods to control the release and be able to reduce the diffusion rate. As discussed in the previous section, the structure of PVA is greatly influenced by the processing parameters. Therefore, it is possible to alter the release rate by adjusting the processing conditions [12].

Several studies have been conducted on PVA hydrogel to demonstrate its utility as a controlled release matrix. These investigations commonly used protein as a model drug, namely BSA [13-15, 152]. Li *et al* studied the release of BSA from PVA hydrogel nanoparticles by the freeze-thaw method. The authors found the release rate of BSA decreased with increasing number of freeze-thaw cycles [13]. This trend has also been confirmed by other groups [14, 15]. Moreover, previous work from our laboratory has revealed that a faster freezing rate or a faster thawing rate will increase the release rate of BSA [12]. Hickey *et al* considered the effect of the number of freeze-thaw cycles on the mesh size of the polymer network and the resulting effect on the diffusion coefficient of solutes. When the crystalline fraction was increased by the freeze-thaw process used, the mesh size decreased resulting in a reduced diffusion coefficient [135].

## **2.7 MOTIVATION FOR THESIS**

Although DES have overcome some difficulties, such as elastic recoil and negative remodeling, they still suffer from in-stent restenosis. The currently used devices have poor control of drug release and use drugs which may do more harm than good. If the drug loading is reduced often there is insufficient delivery resulting in lack of clinical effects [21]. However, if the amount of drug delivered is increased there is a risk of the drug concentration being too toxic or powerful. The coating materials are not easily adjustable to regulate their diffusive properties. The only adjustable features are the addition of a top coat or using variable drug loadings. One of the main downfalls of current DESs is the use of drugs that are non-specific to cell type. Paclitaxel and

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sirolimus inhibit both SMCs and ECs [6]. This causes impaired healing and delayed re-endothelialization, leading to late-stent thrombosis [7, 8, 22, 101]. To minimize late thrombosis the patient is forced on dual anti-platelet therapy for an extended period of time [6, 158, 159].

Our approach to improving upon the current technologies involves using PVA to deliver Serp-1 protein locally to the site of vascular injury. PVA hydrogels provide a hydrophilic matrix that offers advantages over hydrophobic materials by having a reduced thrombogenic potential and having the ability to load protein drugs. Our system also offers tunable diffusion properties just by varying the freeze-thaw processing conditions. The advantage of this is that the same material can be used but is easily adjusted. The release kinetics can also be changed to zero-order with the addition of a second layer of PVA. Again, no additional materials are required, PVA is used for both layers and the processing conditions can give each layer different diffusion properties. Finally, our approach considers the use of a new protein drug, Serp-1. Its main action gives anti-inflammatory properties. By having a different biological target than other drugs considered, this provides a novel approach for the control of restenosis.

This thesis demonstrates work done to examine the diffusion properties of PVA hydrogel using BSA as a model protein. The difference in release kinetics of BSA from single layered (matrix) PVA hydrogel versus multi-layered (reservoir) PVA hydrogel is also observed. Then the release of the therapeutic agent, Serp-1, is characterized and the release kinetics are modelled.

## Chapter 3

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# MATERIALS AND METHODS

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### 3.1 MATERIALS

PVA with a molecular weight ( $M_w$ ) of 146,000-186,000 and 99 +% hydrolysis was obtained from Sigma Aldrich (St. Louis, MO). Bovine serum albumin (BSA) was purchased from Fisher Scientific (Fair Lawn, NJ) and the Protein Dye Reagent Concentrate (cat# 500-0006) for assaying BSA concentration was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA).

Viron Therapeutics Inc. (London, ON) kindly donated all Serp-1 protein and antibodies (ATD11 and bAXB7.9) used for enzyme-linked immunosorbent assay (ELISA) capture and detection. Other reagents for the Serp-1 ELISA included calcium carbonate buffer capsules purchased from Sigma Aldrich (St. Louis, MO), horseradish peroxidase (HRP) streptavidin (cat# P21124) purchased from Pierce Biotechnology, Inc. (Thermo Fisher Scientific), tetramethylbenzidine (TMB) substrate kit (cat# DY999) purchased from R&D Systems (Minneapolis, MN), and reagent grade, 18 N sulfuric acid ( $H_2SO_4$ ) purchased from Caledon Laboratory Chemicals (Georgetown, ON).

All buffers and solutions were made with distilled deionized water. Tween-20 was obtained from Sigma Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) was prepared using potassium phosphate ( $KH_2PO_4$ ) from Caledon Laboratory Chemicals (Georgetown, ON), potassium chloride (KCl) from EMD Chemicals, Inc. (Darmstadt, Germany), sodium chloride (NaCl) from Caledon Laboratory Chemicals (Georgetown, ON), sodium phosphate dibasic heptahydrate ( $Na_2HPO_4 \cdot 7H_2O$ ) from Sigma Aldrich (St. Louis, MO), which were all ACS reagent grade.

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## **3.2 PREPARATION OF PVA HYDROGEL DELIVERY MATRICES**

### **3.2.1 Preparation of PVA Hydrogel**

PVA solution was prepared by dissolving 8, 10, or 12 wt% of PVA in water. Dissolution was achieved in a reaction kettle that was maintained at 90 °C and outfitted with a reflux condenser. Mechanical mixing at 100 rpm was used to ensure a uniform solution was obtained. The PVA solution was heated and mixed for four hours. The solution was poured into containers and allowed to cool to room temperature. PVA solution was poured into the appropriate mould and sealed for freeze-thaw cycling.

#### **3.2.1.1 Freeze-Thaw Cycling of PVA Samples**

The mould containing the PVA solution was submerged in a heated/refrigerated circulating water bath. The hydrogel samples were created by repeated freeze-thaw cycling. One cycle involves freezing from 20 °C to -20 °C at a set rate and holding at -20 °C for one hour before thawing back to 20 °C at a set rate. Freezing and thawing rates were typically set at 0.1 °C/min, but were also varied between 0.05 °C/min and 0.5 °C/min for some experiments. The number of cycles was varied between one and six depending on the study.

### **3.2.2 Preparation of PVA/BSA Hydrogels**

Fresh 10 wt% PVA solution that was cooled to room temperature was carefully poured and 4.4 g weighed into a 50 mm polypropylene Petri dish. A solution of BSA was pipetted into the PVA solution to obtain 0.1 wt% of BSA. The mixture was well stirred to ensure uniform distribution of the protein. The PVA/BSA solution was allowed to sit at room temperature in the Petri dish mould for a period of three hours to allow any bubbles induced during mixing to rise. The moulds were well sealed in plastic bags and clamped into a custom holder to be submerged in the water bath for the freeze-thaw

cycles, as described above in section 3.2.1.1, to form approximately 1.5 mm thick hydrogel discs.

After the required freeze-thaw cycles were completed, the hydrogels were removed from the moulds and the thickness was measured using a custom-made micrometer. The hydrogel discs were measured five times each and an average taken for the thickness.

### **3.2.2.1 Multi-Layered PVA/BSA Hydrogels**

A 25 mL or 15 mL volume of 10 wt% PVA solution was spread onto a custom aluminum mould (125 x 125 mm) with a thickness of either 1.6 mm or 0.8 mm. The mould was closed and then subjected to two freeze-thaw cycles as described above in section 3.2.1.1. This layer was protein-free and creates an outer layer of PVA to act a coating barrier to diffusion.

The mould was opened for a second layer to be added, but the thickness of the first layer was measured. Ten measurements were taken at random places on the hydrogel sheet and averaged for the thickness. The dimensions of the sheet were also measured.

Fresh PVA solution that was cooled to room temperature was carefully poured and weighed into a plastic container. A solution of BSA was pipetted into the PVA solution to obtain 0.1 wt% of BSA. The mixture was well stirred to ensure uniform distribution of the protein. A 25 mL volume of this PVA/BSA mixture was spread directly onto the surface of the PVA layer of two freeze-thaw cycles to create the second layer of approximately 1.5 mm thickness. The mould was closed and further subjected to one more freeze thaw cycle.

Following the freeze-thaw cycle, the result was a two-layer hydrogel sheet. One layer of PVA of three cycles and one layer of PVA/BSA of one cycle. The thickness and

dimensions measurements were repeated again with the two-layer sheet. Then three 40 mm discs were punched from the sheet to be used for the controlled release experiments.

### **3.2.3 Preparation of PVA/Serp-1 Hydrogels**

Fresh PVA solution that was cooled to room temperature was carefully poured and 4.4 g weighed into a 50 mm polypropylene Petri dish. Serp-1 was pipetted into the PVA solution to obtain 0.001 wt% of Serp-1. This amount was varied when studying the effect of Serp-1 loading. The mixture was gently stirred to ensure uniform distribution of the protein. The PVA/Serp-1 solution was allowed to sit at room temperature in the Petri dish mould for a period of three hours to allow any bubbles induced during mixing to rise. The moulds were well sealed in plastic bags and clamped into a custom holder to be submerged in the water bath for the freeze-thaw cycles as described above in section 3.2.1.1 to form approximately 1.5 mm thick hydrogel discs.

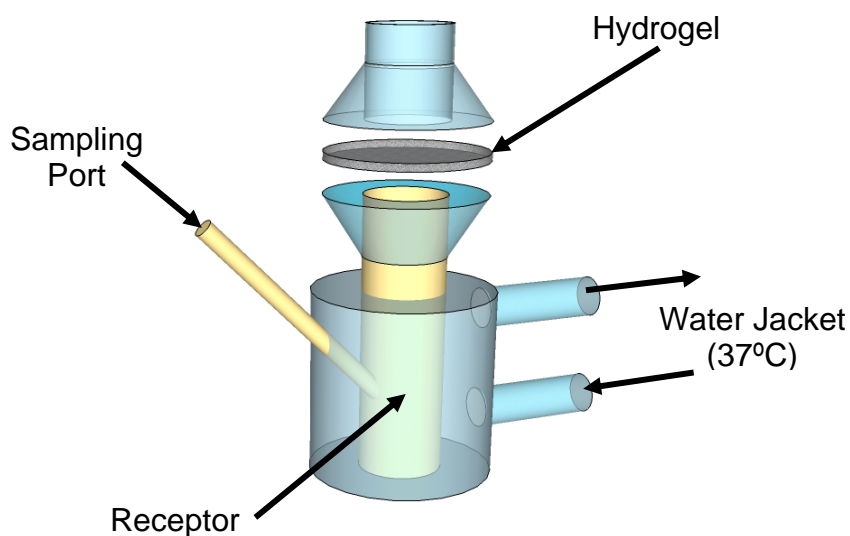
After the required freeze-thaw cycles were completed, the hydrogels were removed from the moulds and the thickness was measured using a custom-made micrometer. The hydrogel discs were measured five times each and an average taken for the thickness. Additionally, the mould was rinsed with 1 mL of assay buffer, which was retained for the ELISA. This was to account for any Serp-1 that may have been squeezed out of the hydrogel during processing.

## **3.3 CONTROLLED RELEASE STUDIES**

### **3.3.1 Franz Diffusion Cells**

The majority of the *in vitro* controlled release studies were done using Franz diffusion cells (FIGURE 3.1). These are vertical diffusion cells with a donor chamber that sits above a receptor chamber and a membrane is typically clamped between the two chambers for permeation studies. For our use, the diffusion cells were modified by

clamping the hydrogel between the two chambers and not actually using the donor chamber since the molecule of interest was previously imbedded into the hydrogel matrix. This allowed for diffusion to occur from the bottom surface of the hydrogel in one direction into the receptor. The 25 mm diameter opening of the diffusion cell provided the area for diffusion to occur.



**FIGURE 3.1.** Schematic of the Franz diffusion cell apparatus. The hydrogel containing drug is clamped in between the receptor chamber and the top allowing diffusion to occur in one direction into the receptor.

The receptor chamber was filled with 20 mL of water or buffer for the release medium. The release medium was magnetically stirred and maintained at 37 °C by a water jacket. The PVA hydrogel samples containing protein were mounted on the diffusion cells with the bottom in direct contact with the release medium and they were wrapped with parafilm to prevent drying. Samples of the release medium were taken at selected time points by temporarily removing the hydrogel sample and pipetting a known

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volume out of the receptor. The release medium was replenished with an equal volume of fresh solution each time and the hydrogel sample returned to its position.

### **3.3.2 Controlled Release of BSA from PVA**

#### **3.3.2.1 Model Protein Release Studies**

PVA hydrogels containing BSA were prepared as described above in section 3.2.2. Six samples were prepared and each one was subjected to one up to six freeze-thaw cycles. The hydrogel samples were mounted in the diffusion cells and with water as the release medium. The release studies were conducted by sampling 5 mL of the release medium at selected time points over a 60 hour period. The samples of release medium were collected for protein detection using ultraviolet-visible (UV-vis) spectroscopy (see section 3.4.1). Once the protein concentration in each sample was determined, the release profile was generated by plotting the cumulative release versus time. The experiments were repeated three times for each sample and the average release curves were plotted.

#### **3.3.2.2 Release from Multi-Layered Samples**

Multi-layered PVA hydrogels containing BSA were prepared as described above in section 3.2.2.1. Two samples were created with different outer layer thickness, 1.6 mm or 0.8 mm. The hydrogel samples were mounted in the diffusion cells and with water as the release medium. The release studies were conducted by sampling 5 mL of the release medium at selected time points over a 500 hour period. The samples of release medium were collected for protein detection using UV-vis spectroscopy (see section 3.4.1). Once the protein concentration in each sample was determined, the release profile was generated by plotting the cumulative release versus time. The experiments were repeated three times for each sample and the average release curves were plotted.

### **3.3.2.3 Release Mediums of Different Ionic Strengths**

PVA hydrogels containing BSA were prepared as described above in section 3.2.2. Four samples were prepared and each one was subjected to two freeze-thaw cycles. The hydrogel samples were mounted in the diffusion cells and with water or PBS as the release medium. Different ionic strengths of release medium was used for each sample, 0X (water), 0.5X, 1X, and 2X. Another two PVA/BSA hydrogel samples were prepared the same as before with the exception that 1X PBS was used as the solvent instead of water. For these two samples the release studies were carried out with a release medium of water or 1X PBS.

The release studies were conducted by sampling 5 mL of the release medium at selected time points over a 140 hour period. The samples of release medium were collected for protein detection using UV-vis spectroscopy (see section 3.4.1). Once the protein concentration in each sample was determined, the release profile was generated by plotting the cumulative release versus time. The experiments were repeated three times for each sample and the average release curves were plotted.

### **3.3.3 Controlled Release of Serp-1 from PVA**

PVA hydrogels containing Serp-1 were prepared as described above in section 3.2.3. Six samples were prepared with 10 wt% PVA and each one was subjected to one up to six freeze-thaw cycles. Three additional samples were prepared with 8 wt%, 10 wt%, and 12 wt% PVA and subjected to two freeze-thaw cycles each. Two further samples were prepared with 10 wt% PVA, and two freeze-thaw cycles with a thawing rate of 0.1 °C/min and a freezing rate of either 0.15 °C/min or 0.1 °C/min. Three more samples were prepared with 10 wt% PVA, and two freeze-thaw cycles with a freezing rate of 0.1 °C/min and a thawing rate of either 0.5 °C/min or 0.1 °C/min or 0.05 °C/min. Another three samples were prepared with 10 wt% PVA and two freeze-thaw cycles, but with varying Serp-1 content of 0.010 wt%, 0.005 wt%, and 0.001 wt%.

The hydrogel samples were mounted in the diffusion cells and with PBS containing 1 % BSA and 0.025 % Tween-20 as the release medium. The release studies were conducted by sampling 5 mL of the release medium at selected time points over a 50 hour period. The samples of release medium were collected for protein detection using ELISA (see section 3.4.2). Once the protein concentration in each sample was determined, the release profile was generated by plotting the cumulative release versus time. The experiments were repeated three times for each sample and the average release curves were plotted.

### **3.3.3.1 Therapeutic Serp-1 Loading**

PVA/Serp-1 samples were prepared by adding 300  $\mu$ L of 17.2 mg/mL Serp-1 to a solution of 10.9 wt% PVA (3.45 g). After mixing in the protein, the final PVA concentration was 10 wt%. The materials were mixed in a 50 mm polypropylene Petri dish and then subjected to two freeze-thaw cycles. After cycling, the hydrogel was then removed from the Petri dish and three 10 mm discs were cut from the solid sample, each with a thickness of approximately 1.2 mm. The theoretical loading of each 10 mm disc was about 206  $\mu$ g of Serp-1. These three discs were each placed in 20 mL of PBS containing 1 % BSA and 0.025 % Tween-20 as the release medium in a polypropylene tube and put into a shaker bath at 37°C. The release medium was sampled at time intervals to determine the concentration of Serp-1 present by ELISA (see section 3.4.22). Once the protein concentration in each sample was determined, the release profile was generated by plotting the cumulative release versus time as an average of the three samples.

### **3.3.3.2 Release into Human Whole Blood**

PVA/Serp-1 samples were prepared by adding 300  $\mu$ L of 17.2 mg/mL Serp-1 to a solution of 10.9 wt% PVA (1.725 g). After mixing in the protein, the final PVA concentration was 10 wt%. The materials were mixed in one well of a polystyrene tissue

culture plate (6 well) with a diameter of 35 mm under aseptic conditions and then subjected to two freeze-thaw cycles. After cycling, the hydrogel was then removed from the 6 well plate and two 10 mm discs were cut from the solid sample, each with a thickness of approximately 1.2 mm. The theoretical loading of each 10 mm disc was about 206  $\mu\text{g}$  of Serp-1. A healthy volunteer was used to collect human whole blood in the presence of the anti-coagulant ethylenediaminetetraacetic acid dipotassium salt ( $\text{K}_2\text{EDTA}$ ). Two of these discs were each placed in 10 mL of human whole blood in a polypropylene tube and put into a shaker at 37 °C. The blood was sampled at time intervals to determine the concentration of Serp-1 present. The samples were processed to obtain the plasma and then frozen until the ELISA was done. Once the protein concentration in each sample was determined, the release profile was generated by plotting the cumulative release versus time as an average of the two samples.

### **3.3.3.3 Serp-1 Release in Different Vessels and Different Buffers**

Two simple release experiments were performed to determine the best method of conducting the Serp-1 controlled release experiments. The first experiment was done by comparing release vessels with either a diffusion cell or a polypropylene tube in a shaker bath. A PVA/Serp-1 sample was prepared as described above in section 3.2.3, using 10 wt% PVA and two freeze-thaw cycles. The hydrogel sample was mounted in a diffusion cell and with PBS containing 1 % BSA and 0.025 % Tween-20 as the release medium. The release studies were conducted by sampling 5 mL of the release medium at selected time points over a 50 hour period. A second PVA/Serp-1 sample was prepared similarly and then cut to a 10 mm disc. This sample was placed in 10 mL of the same release medium in a polypropylene tube which was kept in a shaker bath at 37 °C. The release medium (2 mL) again was sampled at selected time points over a 50 hour period. The samples of release medium were collected for protein detection using ELISA (see section 3.4.2). Once the protein concentration in each sample was determined, the release profile was generated by plotting the cumulative release versus time.



The second experiment by comparing different release mediums in the diffusion cell. Two samples were prepared with 10 wt% PVA and two freeze-thaw cycles. They were mounted in diffusion cells, one with PBS as the release medium and the other with PBS containing 1 % BSA and 0.025 % Tween-20 as the release medium. The release studies were conducted by sampling 5 mL of the release medium at selected time points over a 50 hour period. The samples of release medium were collected for protein detection using ELISA (see section 3.4.2). Once the protein concentration in each sample was determined, the release profile was generated by plotting the cumulative release versus time.

#### **3.3.3.4 Serp-1 Remaining in PVA Matrix**

PVA/Serp-1 samples were prepared by adding 110  $\mu$ L of 17.2 mg/mL Serp-1 to a solution of 10 wt% PVA (4.4 g). The materials were mixed in a 50 mm polypropylene Petri dish and then subjected to two freeze-thaw cycles. Three samples were fabricated, but one was kept for a 0 h time point as a fully loaded matrix. The other two hydrogel samples were mounted in the diffusion cells and with PBS containing 1 % BSA and 0.025 % Tween-20 as the release medium. The release studies were conducted by sampling 5 mL of the release medium at selected time points. One hydrogel sample was removed and stored after 12 h. The other was run for 48 h before completing the study. The three PVA hydrogel samples were stored in the fridge until further study by Western blot (See section 3.4.3) to determine the amount of Serp-1 remaining in the matrix.

The samples of release medium were collected for protein detection using ELISA (see section 3.4.2). Once the protein concentration in each sample was determined, the release profile was generated by plotting the cumulative release versus time.

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## 3.4 PROTEIN QUANTIFICATION

Two methods were used to detect protein concentration in the release medium. For BSA the Bradford method was used to bind a dye to the protein and the colour change was distinguished by measuring the absorbance with UV-vis spectroscopy. For Serp-1 a sandwich ELISA was performed using antibodies to capture and detect the active form of Serp-1.

### 3.4.1 UV-Visible Spectroscopy

The Bradford method was used to add an acidic dye reagent to the BSA release samples. In 15 mL tubes, one part dye reagent concentrate was added to four parts of the BSA release samples. If need be, the samples were diluted with water and the appropriate amount of dye added. The linear region of the assay is 1.2 to 10.0  $\mu\text{g/mL}$ . The tubes were gently shaken to mix and left to incubate at room temperature for 15 min. Samples were then transferred to a 1 cm cuvette. The absorbance of each sample was measured at 595 nm on a Beckman Coulter, DU520, UV-visible spectrophotometer. Three readings for each sample were taken. The absorbance was compared to a calibration curve to determine protein concentration.

### 3.4.2 Enzyme-Linked Immunosorbent Assay (ELISA)

A sandwich ELISA was used to detect Serp-1 in the release samples. The protocol used was inherited from Viron Therapeutics standard operating procedures and details can be seen in Appendix A.

Briefly, samples and standards were added to a polypropylene 96 well plate for dilutions. The assay range is 2 to 24 ng/mL. A standard curve was generated in the first two columns of the 96 well plate by diluting purified Serp-1, 200 ng/mL, in assay buffer

from 0 to 24 ng/mL. In the remaining columns, the samples were serially diluted into the assay range. All samples and standards were run in duplicate.

For the assay, a polystyrene 96 well plate was used. Each well was first coated with the coat antibody in calcium carbonate buffer and the plate was incubated in the fridge at 4 °C overnight. The coat buffer was removed and blocking buffer was added to the wells and allowed to incubate at room temperature for one hour. The plate was washed three times with wash buffer. Then the standards and samples were transferred from the dilution plate to the assay plate and allowed to incubate at room temperature for one hour. The plate was washed three times with wash buffer. The biotinylated detection antibody was diluted in assay buffer and added to each well, incubating at room temperature for one hour. The plate was washed three times with wash buffer. HRP streptavidin was diluted in assay buffer and added to each well, incubating at room temperature for one hour. The plate was washed three times with wash buffer. Equal portions of substrate A and substrate B were mixed from the TMB substrate kit. This mixture was dispensed into the wells and incubated at room temperature for only four minutes. At this time a stop solution of 2 N H<sub>2</sub>SO<sub>4</sub> was added to each well. The plate was then immediately placed in the plate reader and the optical density for each well was read at 450 nm using a Bio-Tek Instruments EL307C manual microplate reader. The data was transferred to an Excel spreadsheet and the concentration of each sample was determined using the standard curve generated on each plate. Only those values in the linear region of the standard curve were used.

#### **3.4.2.1 Serp-1 Activity**

The activity of Serp-1 is also measured by ELISA. The ELISA uses antibodies that are specific to the active form of Serp-1. Therefore, for studies where the activity was of interest, an ELISA was performed and the resulting concentration of each sample compared to a control. Similar concentrations are representative of similar activity levels. This was performed to determine if Serp-1 maintains its activity through the freeze-thaw cycles used for PVA processing and also to compare Serp-1 activity in different buffers.

### 3.4.3 SDS-PAGE and Western Blot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were used to determine the amount of Serp-1 remaining in the PVA matrix. The PVA hydrogels were collected after selected time points (12 h and 48 h) from a controlled release study and a fully loaded matrix (0 h) was also prepared. The hydrogels were melted back into liquid form by heating above 70 °C in a round bottom flask equipped with a mechanical mixer. Once in the liquid form, the PVA samples were diluted with water and prepared for the SDS-PAGE.

The 0 h sample was diluted 16X and 64X and the 12 h and 48 h samples were diluted 8X and 32X in water. 40 µL of each dilution was added to 15 µL of 4X SDS-PAGE sample buffer. The samples, controls (Serp-1, 5 µg/mL) and standard marker were loaded into the gels as shown in TABLE 3.1 for electrophoresis. Further detailed methods can be seen in Appendix B.

**TABLE 3.1.** Gel loading for SDS-PAGE.

Gel 1										
Lane	1	2	3	4	5	6	7	8	9	10
Sample	Bio-Rad Standard	0 h (64X)	12 h (32X)	48 h (32X)	Serp-1	Serp-1	Serp-1	Serp-1	Serp-1	Serp-1
Volume	10 µL	10 µL	10 µL	10 µL	1 µL	2 µL	5 µL	10 µL	20 µL	30 µL
Amount	--	--	--	--	5 ng	10 ng	25 ng	50 ng	100 ng	150 ng
Gel 2										
Lane	1	2	3	4	5	6	7	8	9	10
Sample	Bio-Rad Standard	0 h (64X)	12 h (32X)	48 h (32X)	0 h (16X)	12 h (8X)	48 h (8X)	Serp-1	Serp-1	Serp-1
Volume	10 µL	20 µL	20 µL	20 µL	5 µL	5 µL	5 µL	5 µL	10 µL	20 µL
Amount	--	--	--	--	--	--	--	25 ng	50 ng	100 ng

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Following SDS-PAGE, a Western blot was run to detect Serp-1 on the gels. The gel was blotted on a nitrocellulose membrane to transfer the protein. The protein was then detected with primary and secondary antibodies. The bands on the blot were visualized by chemiluminescent detection and film development. The film was converted to a digital image with a Bio-Rad Gel Doc system. The bands were then analyzed using ImageJ software to find the relative intensity of each band as determined their size and density. The values of each sample were compared to Serp-1 standards run on the same gel to approximate the amount of Serp-1 present in each sample. Further detailed methods can be seen in Appendix C.

### **3.5 RELEASE KINETICS**

The release kinetics of the controlled release studies of protein from PVA hydrogel were analyzed with empirical and semi-empirical models. The Power Law (EQUATION 2.5) was used to analyze the controlled release behaviour and determine the mechanism of release. The Diffusion Model (EQUATION 2.8 and EQUATION 2.9) was used to determine the diffusion coefficients of each protein from PVA hydrogel.

#### **3.5.1 Release Kinetics of BSA from PVA**

SigmaPlot software was used to perform non-linear regression of the release data. The early time approximation of the Diffusion Model was fitted to estimate the diffusion coefficient,  $D$ .

#### **3.5.2 Release Kinetics of Serp-1 from PVA**

For the Serp-1 release data, non-linear regression was not ideal since most release profiles demonstrated less than 50 % release. Thus, a more basic approach was used. A log-log plot of the release profile was used to observe the Power Law relationship and

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find  $n$ . Linear regression was used to find the slope which is representative of the release exponent. For the Diffusion Model, the cumulative release was plotted versus the square root of time to observe a linear relationship. The slope, equal to  $4^2(D/\pi l^2)$ , of this plot was used to calculate  $D$ .

### **3.6 STATISTICS**

Experiments were repeated three times and data expressed as the mean  $\pm$  standard deviation. Microsoft Excel software was used to calculate the mean and standard deviation. Error bars on the release profiles represent the standard deviation.

## **Chapter 4**

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# **RESULTS AND DISCUSSION**

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The goal of this thesis was to design a system to deliver the Serp-1 viral protein over an extended period of time, whereby the system has tunable properties to adjust the timeframe and dosage. Thermally processed PVA hydrogels were investigated for their tunable controlled release properties. A model protein, BSA, was initially used to study the diffusive properties of PVA. Subsequently, the controlled release of Serp-1 was examined and the kinetics of release analyzed.

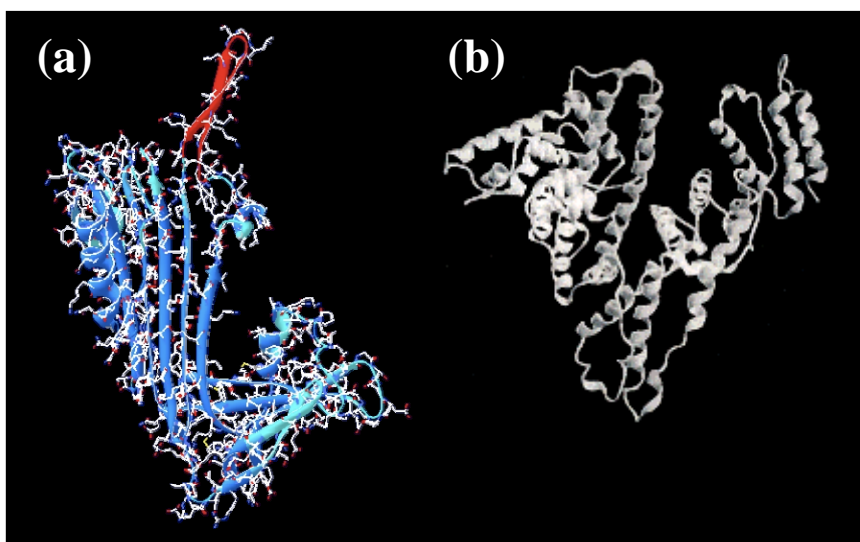
### **4.1 MODEL PROTEIN RELEASE FROM PVA HYDROGEL**

BSA is commonly used for hydrogel controlled release applications as a model protein to demonstrate release properties. We chose to study the release of BSA as a model protein from PVA initially to demonstrate some of the properties of the PVA system for controlled release applications. BSA is readily available, cost effective, and relatively simple to measure making it an ideal model protein. For our particular application, BSA also was chosen for its size of 67 kDa, which is comparable to Serp-1's at 55 kDa (FIGURE 4.1).

Using a model protein that is a similar size to the actual agent of interest, Serp-1, is important for diffusion applications when comparing release rates. The rate of diffusion is influenced by the size of the diffusing molecule and the mesh space available for the molecule to migrate through the mesh. Thus, based on size alone BSA should have a comparable rate of diffusion as Serp-1. However, other factors could contribute to

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a difference in observed release rates. The surface groups on each protein may interact with the PVA hydrogel matrix differently. Serp-1 may have an increased attraction to PVA since it is a glycoprotein and contains sugar groups on its surface. The OH groups from the glycoprotein may hydrogen bond with the OH groups in PVA. If this happens, Serp-1 may have a reduced release rate or reduced amount of release.



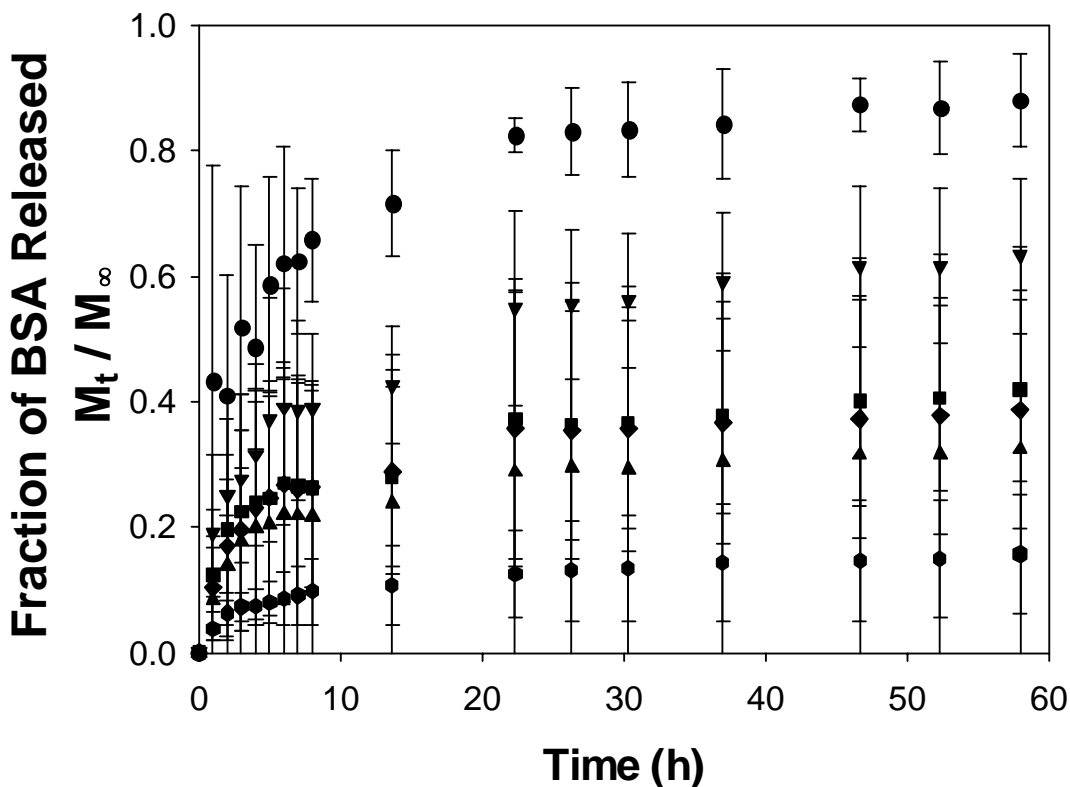
**FIGURE 4.1.** Comparison of therapeutic protein Serp-1 (a) with model protein BSA (b). (a) Courtesy of Jakob Richardson. (b) Reprinted with permission from [160].

#### 4.1.1 BSA Release Profile

Some initial studies were done to investigate the diffusive properties of the PVA system. From the literature we know that the number of freeze-thaw cycles affects the rate of release [13-15, 135]. Adopting a similar experiment using our own lab protocol for the controlled freeze-thaw process, a controlled release profile of BSA from PVA was generated. FIGURE 4.2 demonstrates the control of BSA release as a function of the number of freeze-thaw cycles. With each systematically increasing cycle there is an increase in the local PVA concentration in the polymer rich regions as well as an increase



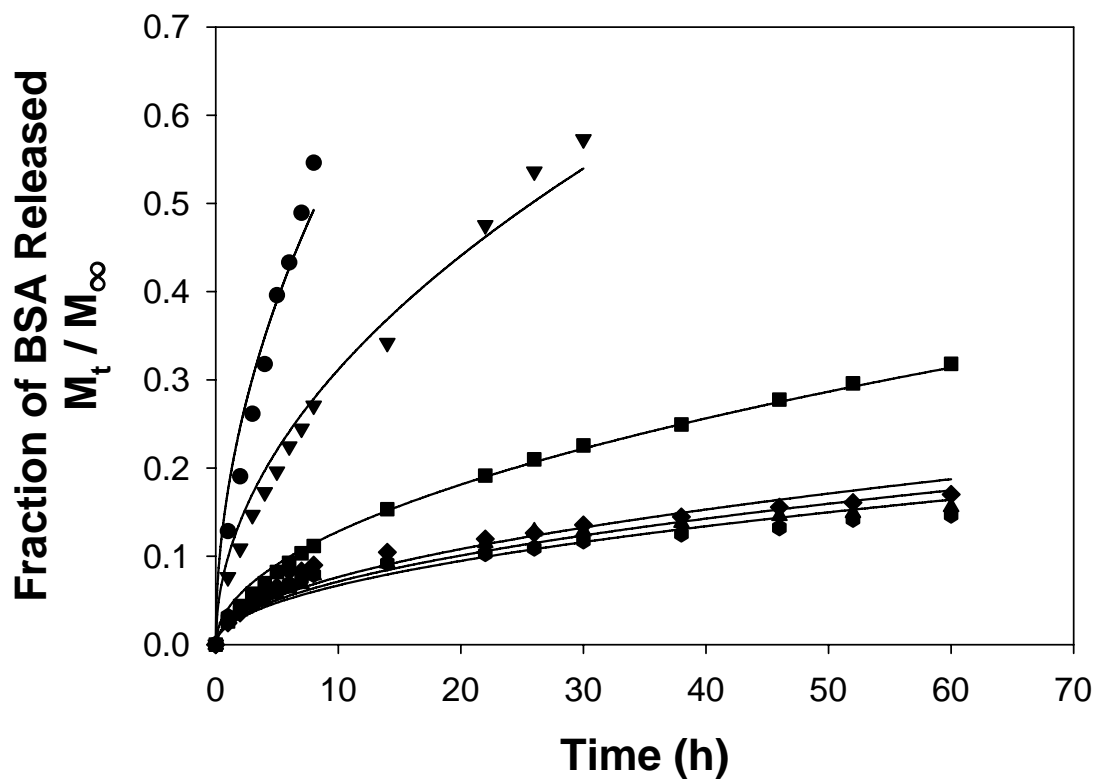
in the volume fraction of the crystalline regions [135]. Thus, the mobility of BSA through the amorphous zones of the polymer rich region is reduced with each cycle leading to the observed decrease in BSA release.



**FIGURE 4.2.** The effect of the number of freeze-thaw cycles on the release of BSA from PVA for 1 cycle (●), 2 cycles (▼), 3 cycles (■), 4 cycles (◆), 5 cycles (▲) and 6 cycles (●). Samples were fabricated with 10 wt% PVA, 0.05 wt% BSA, and both freezing and thawing rates at 0.1 °C/min.

The Diffusion Model (EQUATION 2.8) was fitted to the data to determine the diffusion coefficient for each freeze-thaw cycle. FIGURE 4.3 displays the fit of the model on a representative set of data. Only the early-time approximation was used as it is common to use only the first equation of the model to find the diffusion coefficient and data falling under 60 % release was fitted. TABLE 4.1 is a summary of the results from

the kinetic analysis of the BSA release profiles. The results indicate a decreasing trend in diffusion coefficient,  $D$ , with increasing number of freeze-thaw cycles as expected. The diffusion coefficients fell within a range of  $1.17 \times 10^{-9}$  to  $1.19 \times 10^{-7} \text{ cm}^2/\text{s}$ .



**FIGURE 4.3.** An example of fitting the Diffusion Model using the early-time approximation. Shown are 1 cycle (●), 2 cycles (▼), 3 cycles (■), 4 cycles (◆), 5 cycles (▲) and 6 cycles (●). Samples were fabricated with 10 wt% PVA, 0.05 wt% BSA, and both freezing and thawing rates at 0.1 °C/min.  $R^2$  values for the linear fits are 0.9551 (cycle 1), 0.9817 (cycle 2), 0.9945 (cycle 3), 0.9475 (cycle 4), 0.9603 (cycle 5), and 0.9115 (cycle 6).

**TABLE 4.1.** The effect of the number of freeze-thaw cycles on the release of BSA from PVA. Samples were fabricated with 10 wt% PVA, 0.05 wt% BSA, and both freezing and thawing rates at 0.1 °C/min.

Cycle #	$D_{\text{EARLY}} \pm \text{Std Dev}$ ( $10^{-8} \text{ cm}^2/\text{s}$ )
1	11.9 $\pm$ 6.59
2	3.18 $\pm$ 3.24
3	0.74 $\pm$ 0.53
4	0.85 $\pm$ 0.67
5	0.54 $\pm$ 0.52
6	0.12 $\pm$ 0.11

Comparing these results with those of similar work reported previously from our lab, a similar trend was observed for BSA release from PVA hydrogel as a function of the number of freeze-thaw cycles. The release of BSA was found to decrease with increasing number of cycles and the diffusion coefficients were on the order of  $10^{-8} \text{ cm}^2/\text{s}$  [12]. Therefore, the results presented in TABLE 4.1 are as expected based on previous experience working with the same system in our lab.

Structure of the physically crosslinked PVA hydrogel prepared by thermal cycling has been investigated extensively [144-146] and has been used to elucidate the structure of PVA hydrogel prepared by freeze-thaw processes. It was found that it has a heterogeneous structure with phase separated domains. In the initial freeze-thaw cycle, ice crystals in the amorphous regions force the polymer chains into regions of high local polymer concentration, forming crystallites [138, 145, 147]. Further cycling increases the overall crystallinity by increasing the size of primary crystallites, as well as forming additional smaller secondary crystallites, transforming the microstructure into a fibrillar network. The crystallites have dimensions of about 3 nm and are separated by amorphous regions of around 20 to 30 nm in size within the polymer rich regions. These

polymer rich regions are surrounded by polymer poor regions (macropores) with dimensions of >100 nm [145, 151]. The porous structure arises from the existence of the polymer poor regions which are essentially filled with water. Every thermal cycle after the first freeze-thaw cycle resulted in an increase in the local PVA concentration in the polymer rich regions concomitant with an increase in the volume fraction of the crystalline regions [135]. This would increase the time required for the movement of BSA through the amorphous zones of the polymer rich region resulting in the observed decrease in the release.

#### **4.1.2 BSA Release from Multi-Layered PVA Samples**

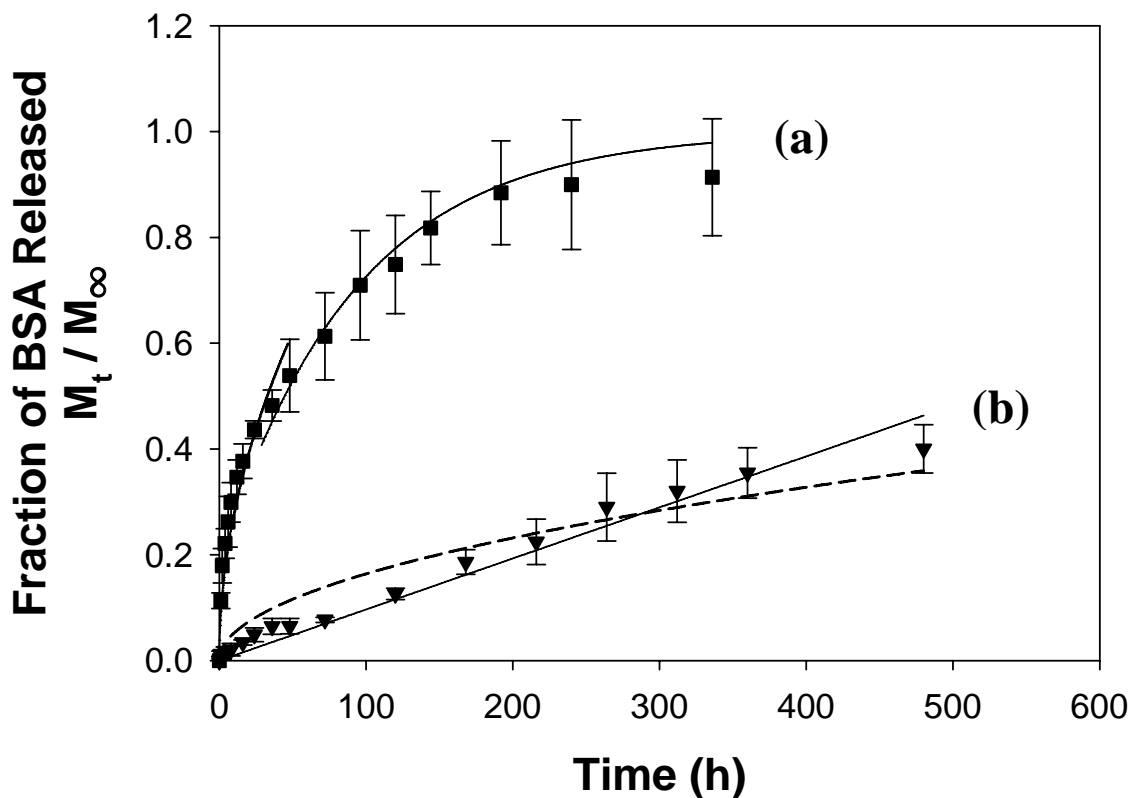
BSA was used as a model protein to further investigate properties of protein release from PVA hydrogels under different configurations. For example, the majority of work published in the literature has demonstrated BSA release from a PVA matrix (monolith). A simple, yet effective way to alter the release kinetics is to add an outer layer to create a reservoir-type system.

##### **4.1.2.1 Matrix versus Reservoir**

BSA release was observed from both a matrix-type system and a reservoir-type system to compare the release kinetics (FIGURE 4.4). The matrix-type system was the typical PVA hydrogel slab used in previous studies and was processed with three freeze-thaw cycles. The reservoir-type system was achieved by adding an additional layer of PVA without protein on a BSA-containing slab to act as a barrier to diffusion. For the system studied, the BSA containing layer was of one freeze-thaw cycle, while the outer BSA free layer was of three cycles. Therefore, the inner BSA layer could be regarded as a reservoir for BSA and should allow relatively free movement of BSA while the outer layer provides more resistance to molecular movement and acts as a rate-controlling barrier.

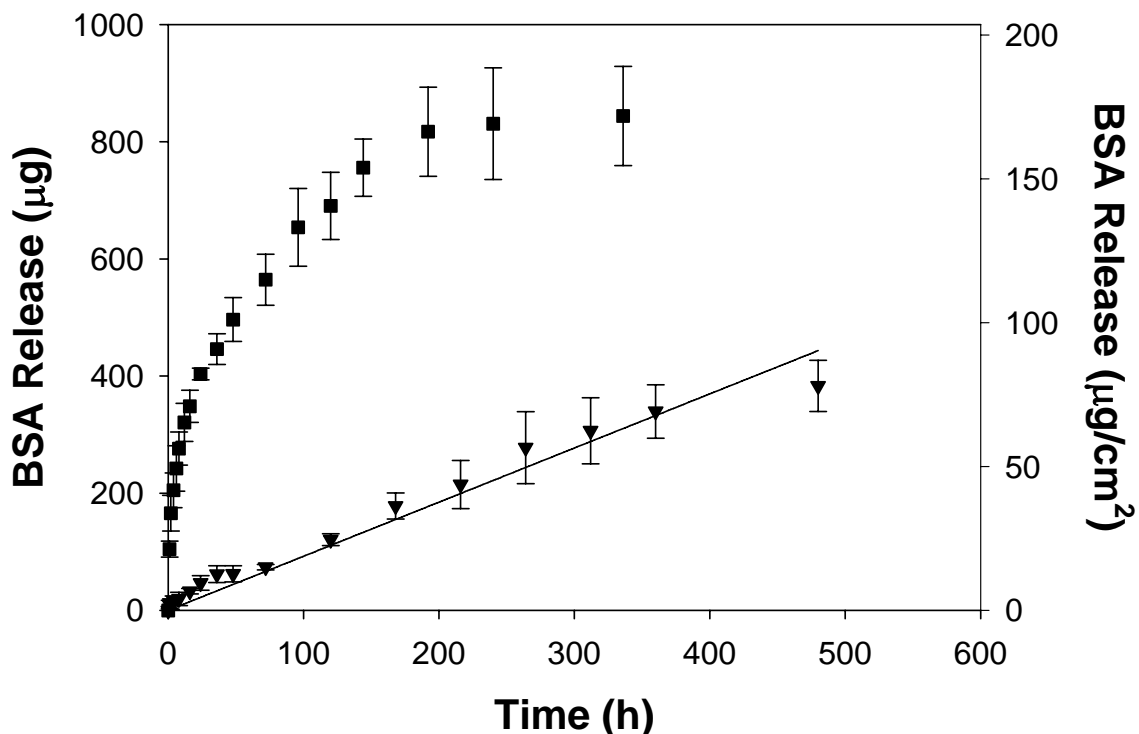
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FIGURE 4.4(a) displays the matrix-type system as a diffusion controlled release system and the Diffusion Model (EQUATION 2.8 and EQUATION 2.9) fit is shown. Also displayed is the reservoir-type system (FIGURE 4.4(b)) showing a more gradual release with close to zero-order kinetics. For comparison the reservoir-type system is shown with both a Diffusion Model (EQUATION 2.8) fit and a linear fit. It is clear that the Diffusion Model ( $R^2=0.9343$ ) does not fit the reservoir-type data well and that a linear fit ( $R^2=0.9679$ ) appears more appropriate. Reservoir-type systems maintain a constant concentration of molecules diffusing across a barrier while matrix-type systems release molecules directly from the surface. Thus, the linear relationship is expected since reservoir-type systems follow zero-order release kinetics, unlike matrix-type systems which follow Fickian diffusion [87]. Both types of systems studied have BSA diffusing through PVA of three cycles, however, the reservoir-type reduces the rate of release since initially there is no BSA in the outer barrier of PVA and the BSA must be released across the barrier from the inner reservoir. Thus, the BSA releases at a constant rate, whereas for the matrix-type the BSA readily diffuses out with the molecules closer to the surface releasing first and the release rate is time dependent, slowing as molecules from the most furthest region take longer to reach the surface.



**FIGURE 4.4.** Fractional BSA release from PVA, comparing (a) matrix-type (■) and (b) reservoir-type (▼) systems. For (a) the Diffusion Model is fit and for (b) the Diffusion Model is fit (dashed line,  $R^2=0.9343$ ) as well as a linear fit (solid line,  $R^2=0.9679$ ). The matrix was fabricated of 10 wt% PVA with 0.1 wt% BSA using 3 freeze-thaw cycles. The reservoir had a drug layer of 10 wt% PVA with 0.1 wt% BSA and 1 freeze-thaw cycle and an outer barrier of 10 wt% PVA of 3 freeze-thaw cycles.

The diffusion coefficient for the matrix-type system is  $1.15 \times 10^{-8} \text{ cm}^2/\text{s}$  determined by fitting the Diffusion Model in FIGURE 4.4. However, for the reservoir-type system, release rate determination requires absolute release amounts. FIGURE 4.5 displays the above data plotted with the actual BSA release amounts instead of fractional release on the y-axis. From FIGURE 4.5 the linear release rate of the reservoir-type system was determined to be  $5.23 \times 10^{-5} \mu\text{g}/\text{cm}^2 \cdot \text{s}$ .



**FIGURE 4.5.** Amount of BSA release from PVA, comparing (a) matrix-type (■) and (b) reservoir-type (▼) systems. The matrix was fabricated of 10 wt% PVA with 0.1 wt% BSA using 3 freeze-thaw cycles. The reservoir had a drug layer of 10 wt% PVA with 0.1 wt% BSA and 1 freeze-thaw cycle and an outer barrier of 10 wt% PVA of 3 freeze-thaw cycles.

The effect of barrier thickness on release rate of the reservoir system is demonstrated in FIGURE 4.6. According to Fick's first law, the flux of a molecule across a membrane is largely dependent on the thickness of the membrane. This thickness is the distance a molecule must migrate across. Consequently, the thicker the layer the longer it will take for the molecule to cross. As demonstrated, when the outer BSA-free barrier layer of PVA was increased in thickness, the release rate of BSA was reduced.

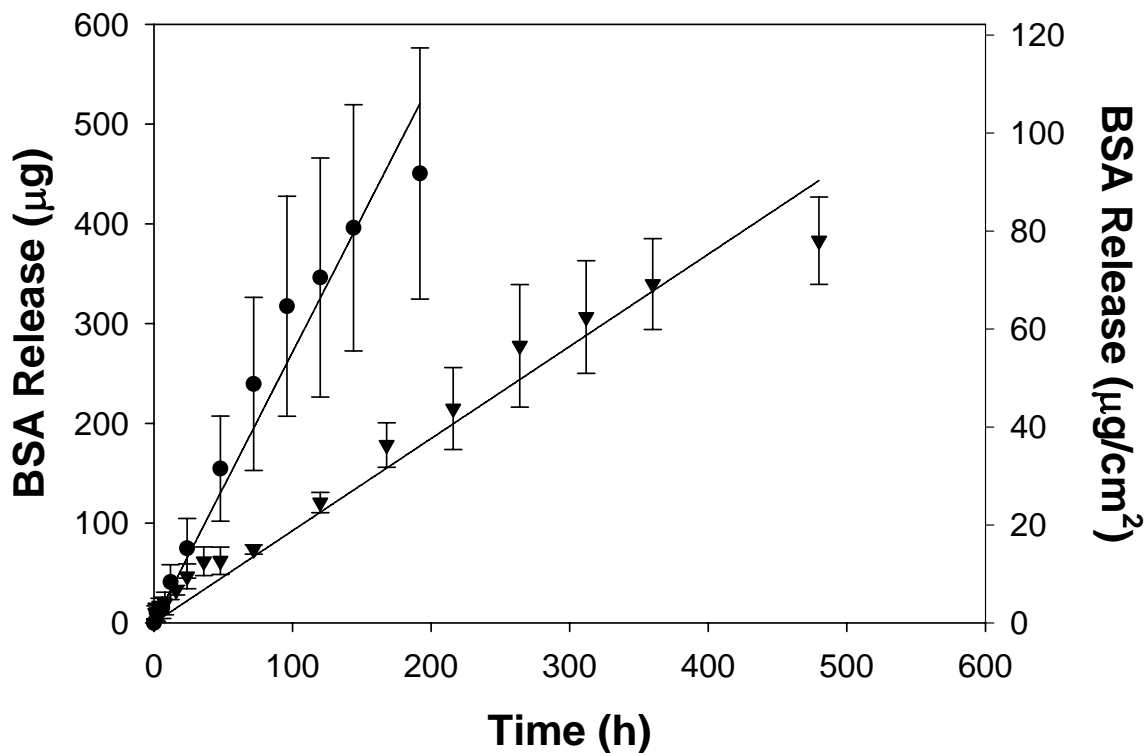
To determine the release rates in the two cases of barrier thickness, the absolute amount of BSA release is again required and is shown in FIGURE 4.6. For a barrier thickness of 0.8 mm the release rate was  $1.53 \times 10^{-4} \mu\text{g}/\text{cm}^2 \cdot \text{s}$  and for a thickness of 1.6

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mm the release rate was  $5.23 \times 10^{-5} \mu\text{g}/\text{cm}^2 \cdot \text{s}$ . The release rate does not scale linearly with barrier thickness. The thicknesses of the barriers differ by a factor of two, but the release rates differ by a factor of about three. Holding all other parameters constant, the release rates should be inversely proportional to the thickness according to Fick's first law (EQUATION 2.1). A potential reason for the release rate not scaling linearly with barrier thickness could be due to mixing at the interface between the two layers. Since the reservoir and the barrier layers are prepared by the freeze-thaw technique consecutively, it is inevitable that some degree of mixing at the interface will occur when the PVA solution is poured onto a previously solidified layer of two freeze-thaw cycles (barrier) to create the one cycle (reservoir) layers. This mixing potentially reduces the thickness of the barrier layer leading to a higher release rate, but its overall effect should decrease with an increased barrier thickness.

Advantages of the multi-layered PVA system include a release rate independent of time and the release rate can be controlled by simply changing the barrier layer thickness. This type of system would especially benefit applications where a constant release rate is required. An example is the release of anti-microbial agents for the control of infection in surface wounds. It would also apply to cases where extended drug availability is needed and the concentration can be adjusted by controlling the initial drug loading.





**FIGURE 4.6.** BSA release from PVA, reservoir-type systems with different coating thicknesses, showing the absolute amount released on the left axis and the amount released per surface area on the right axis. The outer coating thicknesses were 0.8 mm (●) and 1.6 mm (▼).

One distinct advantage of the multi-layer PVA reservoir-type system is that only one type of material was required for its fabrication. And, the layers of PVA easily bond together in the physical crosslinking process, forming two distinct layers of the same material that adhere well. If the two layers do not adhere well, there would be the risk of device failure and the possibility of ‘dose dumping’. This would result in a much faster release of the drug from the inside reservoir layer if the outer barrier layer is defective. Often times a different material from the drug core is used to coat reservoir-type devices. Examples of this include transdermal patches and oral tablets. Delayed release oral tablets typically have an internal drug core containing pore formers such as hydroxy propyl methyl cellulose (HPMC) and poly(ethylene glycol) (PEG). The most common

materials used for the outer membrane are ethylcellulose and acrylic copolymers [89]. Transdermal patches can be multilaminar systems, for instance, Nicoderm is composed of a nicotine-containing ethyl vinyl acetate drug layer and a polyethylene rate-controlling membrane [89]. By using two different materials for the layers, different diffusive properties can be achieved but there would be a higher probability of the interface between the two layers failing by delamination.

Since PVA hydrogel has tunable properties, it is easy to fabricate a system with multiple layers of different diffusive properties by adjusting the processing parameters used for each layer. In the above example, the number of cycles for each layer was varied, but the other processing parameters including freezing and thawing rates in addition to PVA concentration are other options for adjusting the properties of each layer.

### 4.1.3 Effect of Release Medium Ionic Strength

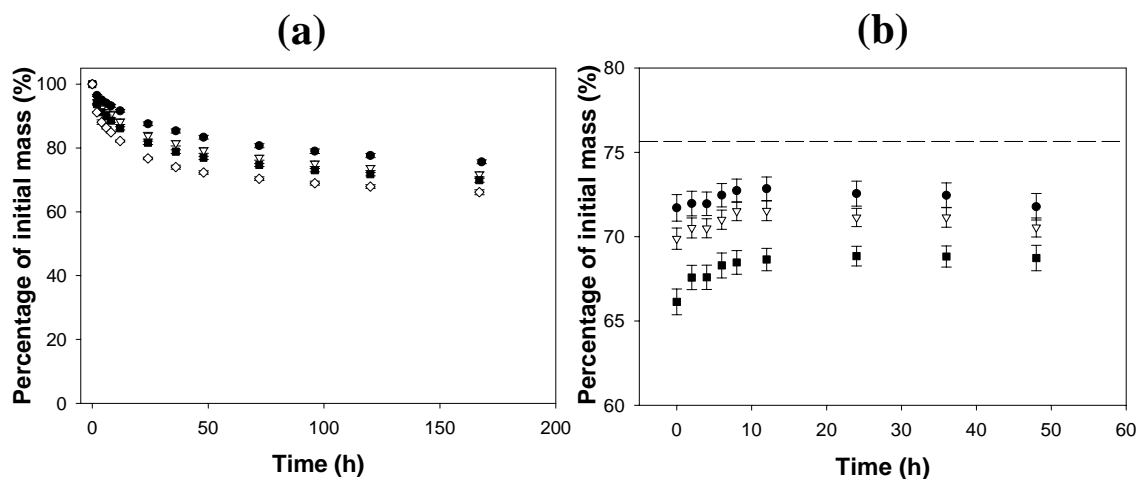
BSA was also used as a model protein to demonstrate release from PVA when an osmotic pressure is induced on the hydrogel by using release mediums of varying ionic strength. An osmotic pressure gradient was created by conducting the controlled release experiments in PBS, while the PVA hydrogel was fabricated with water. Thus, there was a concentration difference of ions inside the hydrogel matrix from outside in the release medium. FIGURE 4.8 illustrates the *in vitro* controlled release profile of BSA from PVA hydrogel when PBS of increasing ionic strength is used as the release medium. As can be seen, the amount of BSA release decreased with increasing PBS ionic strength. The corresponding ionic strengths of PBS used are given in TABLE 4.2.

**TABLE 4.2.** Ionic strengths of PBS used.

	Ionic Strength (mol/L)
0X PBS	0
0.5X PBS	0.09
1X PBS	0.17
2X PBS	0.34

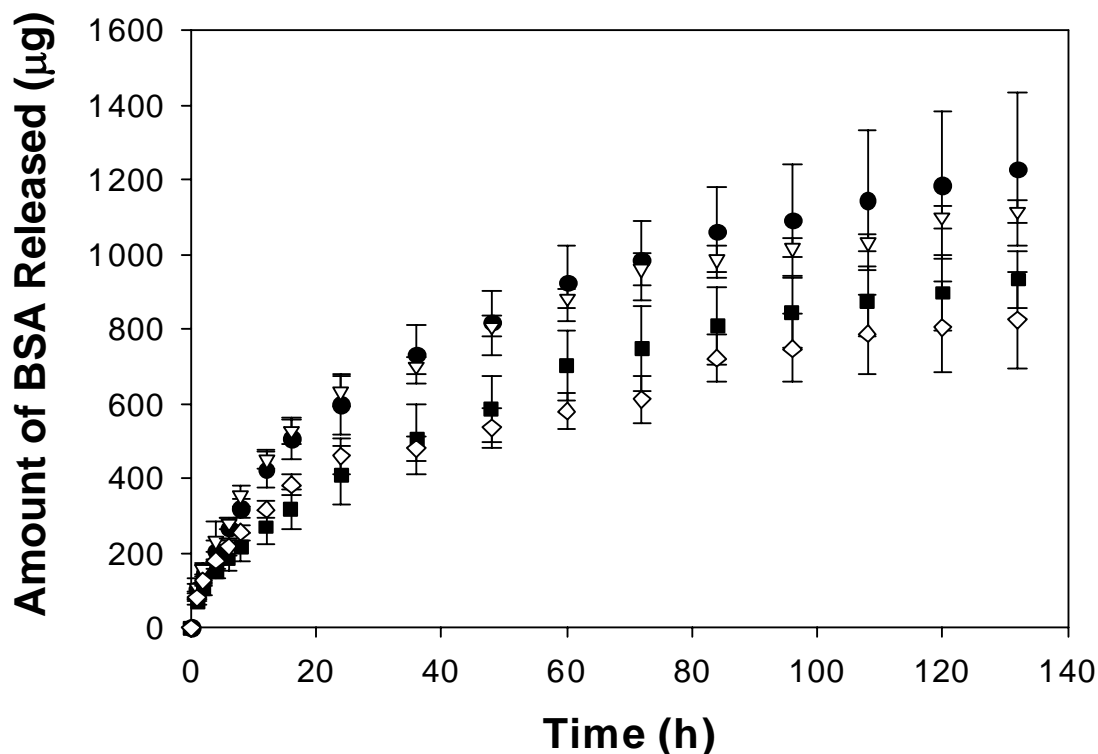
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Osmosis causes the hydrogel to deswell as water moves out of the hydrogel matrix to the release medium of higher ionic concentration in an effort to reach osmotic pressure equilibrium. Patachia *et al* investigated the behavior of PVA hydrogels in electrolyte solutions and determined the mass of the hydrogel decreased over time when placed in solutions containing sodium chloride or potassium chloride. The authors indicated the mass loss was due to water being eliminated through osmosis. The effect was stronger as the concentration of electrolytes in the solution was increased [161]. Effectively the water loss causes a slight collapse in macromolecular structure of the hydrogel matrix. Additional evidence comes from collaborative work with another member in our group who has demonstrated that there is up to 30 % mass loss of PVA hydrogels in PBS of varying ionic strengths (FIGURE 4.7(a)) [162]. Also demonstrated are the reswelling curves of the same PVA hydrogels when placed back in water following shrinkage in PBS. FIGURE 4.7(b) indicates that the samples do not necessarily fully reswell back to their original weight which is denoted by the dashed line. This signifies that the osmotically induced shrinkage of PVA hydrogels can be, at least partially, irreversible [162]. FIGURE 4.7 illustrates the changes in mass for PVA samples of two freeze-thaw cycles. Further work with PVA samples of six freeze-thaw cycles exhibits a similar trend, however the effect is not as pronounced [162]. Hence, the effect of osmotic pressure will be less on stiffer PVA hydrogels created with increased number of freeze-thaw cycles.



**FIGURE 4.7.** (a) Mass loss of 10 wt% PVA hydrogels with two freeze-thaw cycles after one week in distilled water and PBS of varying ionic strengths, water or 0X PBS (●), 0.5X PBS (▽), 1X PBS (■), 2X PBS (◇). (b) Re-swelling of 10 wt% PVA hydrogels with two freeze-thaw cycles in distilled water after one week in 0.5X PBS (●), 1X PBS (▽), 2X PBS (◇). Dashed line indicates mass percent of 10 wt% PVA hydrogel after one week of soaking in distilled water. Printed with permission from [162].

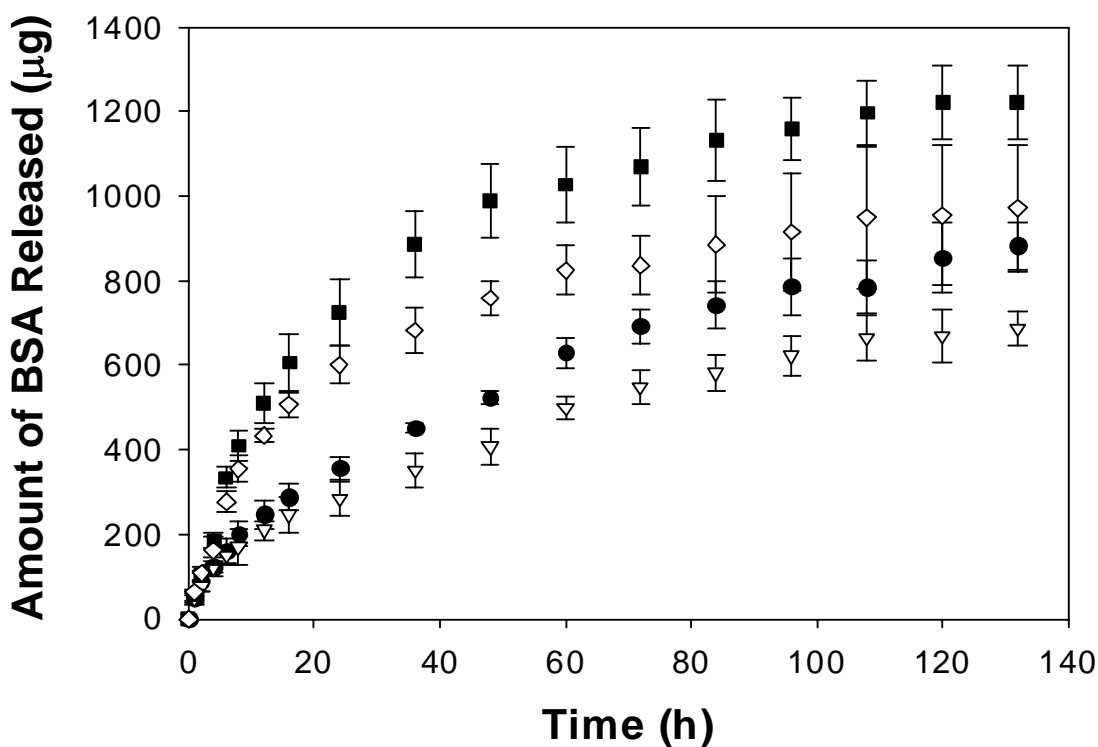
The shrinkage of the structure affects the release rate since the release is dependent on the ability of the protein to be able to move through the mesh of the polymer network, which is largely contingent on the degree of crosslinking, crystallinity and the mesh space available for diffusion [163-165]. Given that protein release from PVA hydrogels is known to be by a diffusion process, the mesh space available for the protein's mobility plays a major role in the release rate. The BSA has to diffuse across a relatively higher crystalline density and one of higher PVA content due to the water loss. Therefore, with the increased ionic strength of the release medium, there is less space for diffusion of BSA and some becomes permanently trapped in the PVA matrix leading to the observed decrease in released amount.



**FIGURE 4.8.** Controlled release profile of BSA (0.1 wt%) from 10 wt% PVA into PBS of varying ionic strength, water or 0X PBS (●), 0.5X PBS (▽), 1X PBS (■), 2X PBS (◇). Error bars represent the standard deviation with  $n=3$ .

A second study was implemented to determine if reducing the osmotic pressure by changing the PVA solvent to PBS could affect the BSA release. FIGURE 4.9 displays four possible scenarios. Samples fabricated by dissolving PVA in PBS and releasing BSA into either water or PBS. And, samples fabricated by dissolving PVA in water and releasing BSA into either water or PBS. For PVA samples dissolved in the same solvent used for the release medium, the osmotic pressure should be alleviated. Yet, it can be seen that PVA in water does not behave exactly the same as PVA in PBS. This is likely due to the presence of ions during the physical crosslinking process which may influence the hydrogen bonding and structural properties of the hydrogel. Of more important

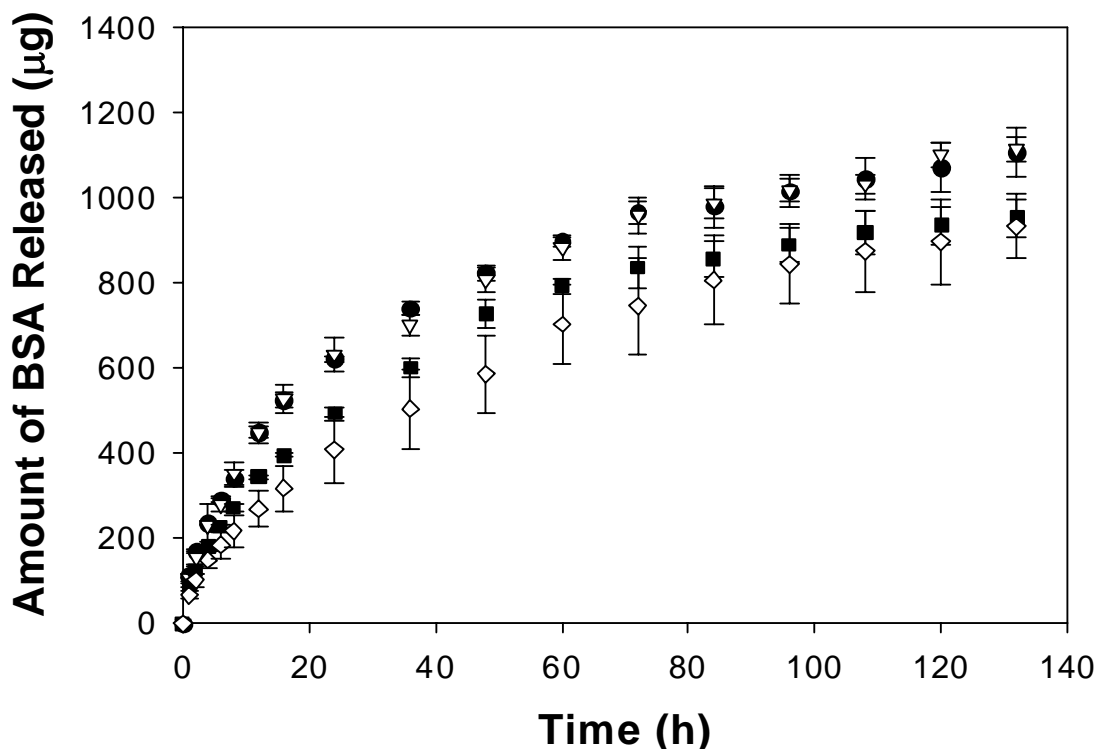
interest are the scenarios where PVA is not dissolved in the same medium as used as the release medium. In FIGURE 4.9, when PVA is dissolved in water and then placed in contact with PBS, the effect is the same as shown in FIGURE 4.8, where the release is decreased due to the osmotic pressure effect. However, when PVA is dissolved in PBS and then placed in contact with water, the observed effect is opposite. In this case, the osmotic pressure gradient is reversed and water will want to move into the hydrogel causing it to swell. This will effectively create more space for diffusion and the rate and amount of release will increase.



**FIGURE 4.9.** Controlled release profile of BSA (0.1 wt%) from 10 wt% PVA, comparing the use of water or PBS as a solvent for PVA. Samples were fabricated by dissolving PVA in PBS and releasing BSA into either water (■) or PBS (◇) and also by dissolving PVA in water and releasing BSA into either water (●) or PBS (▽). Error bars represent the standard deviation with n=3.

One further study was carried out to assess the effect of an increase in effective PVA concentration in the hydrogel by osmotic water loss on BSA release. When placed in a solution of higher ionic strength, the hydrogel deswells as water is lost due to osmotic pressure. This causes an overall effective increase in the PVA content of the hydrogel. PVA concentration is a known parameter that influences the release rate of protein from the hydrogel, where increasing the PVA concentration decreases the release rate [12]. For our study it was predicted the release profile of BSA in a 10 wt% PVA hydrogel matrix into PBS would be similar to that of PVA of a higher concentration into water. From the mass of water loss, it was determined that the PVA concentration should increase to 10.6 wt% over time in 1X PBS. By determining the amount of water lost from the PVA hydrogel when in 1X PBS from when in water, the effective PVA concentration can be determined from a ratio of the initial PVA content (10 wt%) to the percentage of mass loss due to water alone. FIGURE 4.10 shows a comparison of release profiles. With an initial PVA concentration of 10.6 wt% the release into water was found to closely match that of 10 wt% PVA into 0.5X PBS and not 1X PBS. Instead an initial PVA concentration of 10.8 wt% into water was found to more closely resemble 10 wt% PVA into 1X PBS.

As water is lost from the hydrogel it takes some time to reach equilibrium and the deswelling effect is not immediate. Therefore, the PVA concentration will change gradually overtime and would not be possible to match exactly by changing the initial PVA concentration as done in this study. The PVA concentration will initially be lower and gradually increase overtime in PBS which will gradually decrease the released amount of BSA. Thus, varying the initial PVA concentration for release in water would not be an exact match. Nonetheless, this study demonstrated an overall decrease in protein release when the initial polymer concentration is increased. A similar effect is seen where increasing PBS strength eventually increases the polymer concentration, causing a decrease in the amount released. Therefore, there is an equivalence of effects where an increase in PVA concentration and shrinkage of the PVA structure from osmotic pressure decrease BSA release from PVA hydrogel.



**FIGURE 4.10.** Controlled release profile of BSA (0.1 wt%) from PVA into water, 10.6 wt% PVA (●) and 10.8 wt% PVA (■). Curves match closely with BSA (0.1 wt%) release from 10 wt% PVA into 0.5X PBS (▽) and 1X PBS (◇). Error bars represent the standard deviation with n=3.

## 4.2 SERP-1 ACTIVITY

When working with systems containing protein, there are many possible ways a protein may lose its activity, thus it is critical to ensure the protein activity is maintained so the system is effective and efficient. The processing of PVA hydrogels involves repetitive freezing and thawing which can greatly reduce some protein's activity. For that reason, the first step to working with the Serp-1 viral protein was to make certain the protein's activity was maintained through the freeze-thaw cycling. Additionally, when working with protein, its behaviour in different buffers and vessels of different materials



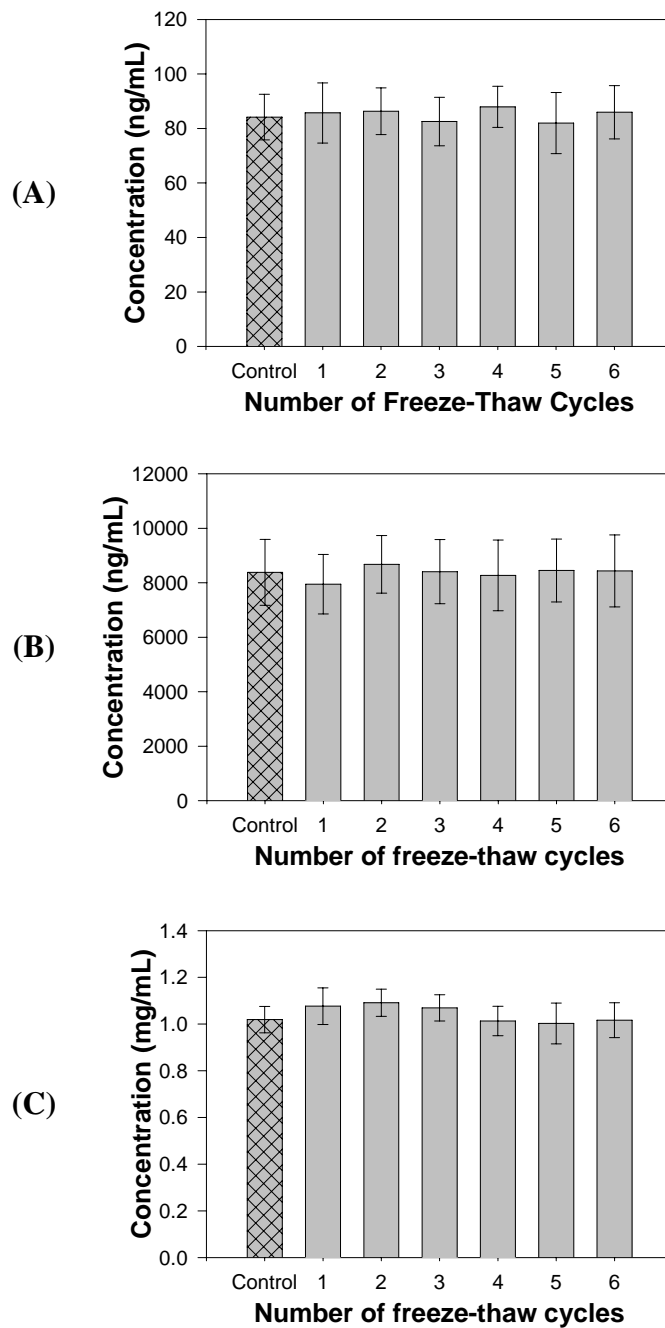
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can affect working concentrations of active protein, particularly due to surface adsorption problems.

#### **4.2.1 Repeated Freezing and Thawing of Serp-1**

Serp-1 protein was subjected to the same controlled freeze-thaw cycles used to process PVA. In this study the protein on its own was tested to ensure its activity was maintained through all six possible cycles. An ELISA was used to measure the concentration of Serp-1 following each cycle and compared to the concentration of a control sample with no freeze-thaw treatment. The ELISA utilized functions by using specific antibodies for the active form of Serp-1. Therefore, not only is it a measure of concentration, but it is also a measure of the activity of the protein as non-active forms will not be detected.

FIGURE 4.11 shows the concentrations measured for cycles one to six for three different starting concentrations of Serp-1, (A) 100 ng/mL, (B) 10,000 ng/mL and (C) 1 mg/mL. When comparing to the concentration of control group in each graph, the activity was maintained through all freeze-thaw cycles for each starting concentration of Serp-1. Consequently, the freeze-thaw cycling does not deactivate the Serp-1 protein. Therefore, it should be reasonable to expect the activity of Serp-1 to be maintained under the PVA processing conditions provided that PVA itself does not denature the protein.

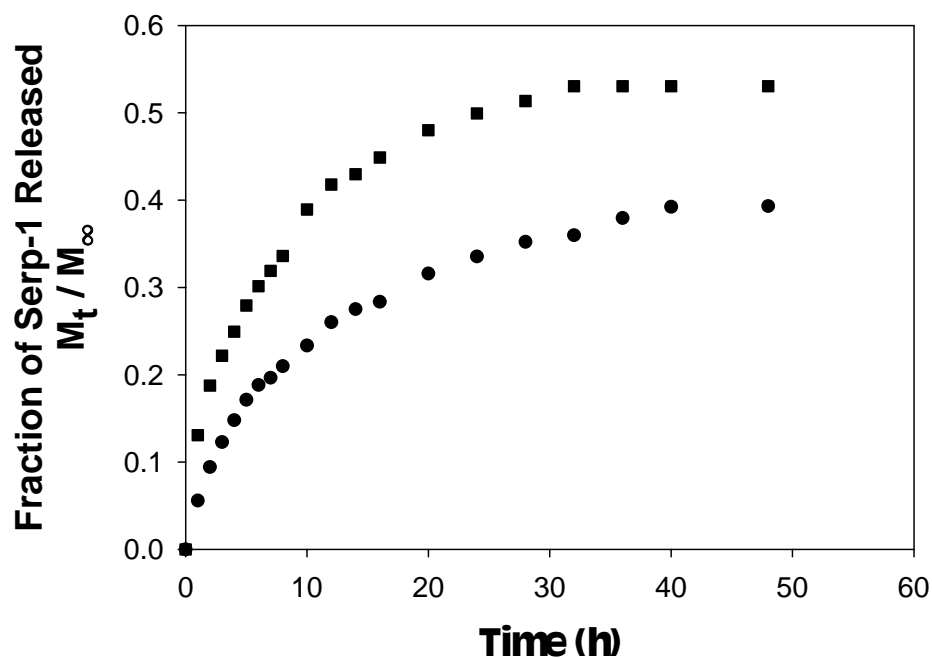


**FIGURE 4.11.** Concentration of Serp-1 following the controlled freeze-thaw cycles (1-6) compared to a control with no freezing and thawing. The concentrations were determined by ELIZA using specific antibodies for active Serp-1 only and thus it is also a measure of activity. A range of starting concentrations are demonstrated for (A) 100 ng/mL, (B) 10,000 ng/mL and (C) 1 mg/mL. Error bars represent the standard deviation with n=3.

### 4.2.2 Serp-1 Behaviour in Vessels of Different Materials

Protein stability and adsorption to the vessel wall in which it is contained can often be a problem when working at low protein concentrations. For our studies, if Serp-1 adsorbs to the wall of the vessel in which the release experiment is conducted, the measurement of the concentration will be lacking the adsorbed amount and the observed kinetics will not be entirely accurate. To test this, a simple release experiment was conducted in two different vessels, a glass diffusion cell and a polypropylene tube. From FIGURE 4.12 it can be seen that the concentration of detected Serp-1 was higher in the polypropylene tube which should not adsorb much protein. The results indicate that protein adsorption is a problem when working with the glass diffusion cells. However, the diffusion cells offer other advantages and are convenient for the *in vitro* release studies, therefore, the next section investigates how to minimize the adsorption while working with the diffusion cells.

These results also help to validate the use of polypropylene dishes as moulds to fabricate the hydrogel samples containing Serp-1. Polypropylene Petri dishes were used to make all the controlled release hydrogel samples in an effort to not lose any of the loaded Serp-1 due to adsorption to the mould during processing.



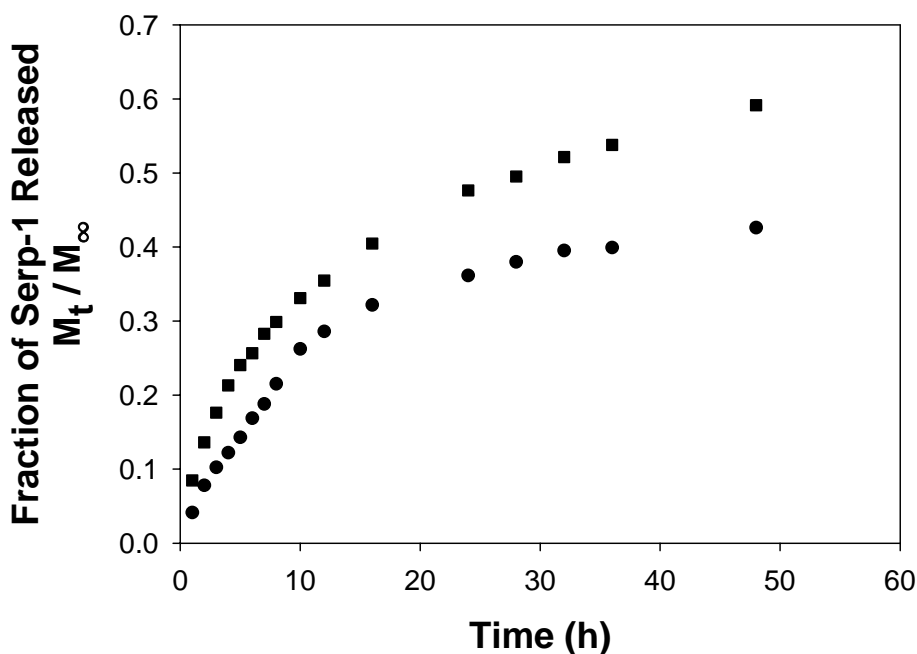
**FIGURE 4.12.** Release profiles of Serp-1 demonstrating the difference in release from different vessels by comparing polypropylene tubes (■) and glass diffusion cells (●).

### 4.2.3 Serp-1 Behaviour in Different Buffers

The *in vitro* controlled release studies were conducted in glass Franz diffusion cells. Serp-1 loss due to adsorption to the diffusion cell surface, as demonstrated in FIGURE 4.12, must be kept under control. One approach to minimize Serp-1 adsorption to the diffusion cell is by competitive pre-adsorption of another inert protein, such as BSA, onto the diffusion cell surface. This approach was evaluated using PBS containing BSA as the release medium.

BSA is often used as an added ingredient in biological mediums to stabilize other protein structure and prevent losses of protein to surfaces. Here, it acts in a competitive manner with Serp-1. If BSA adsorbs to the vessel walls first and the kinetics of adsorption for BSA is favorable, Serp-1 will stay in solution, allowing for accurate determination of Serp-1 concentration. This was investigated by conducting experiments

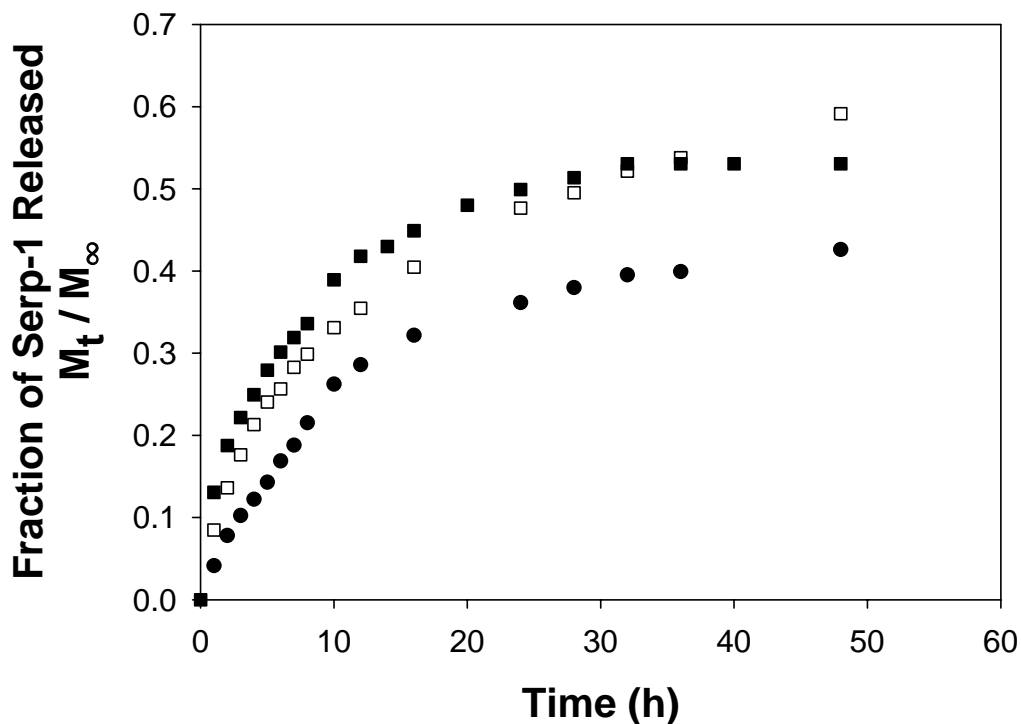
of Serp-1 release from PVA hydrogel into different buffers. FIGURE 4.13 demonstrates a simple *in vitro* release experiment of Serp-1 from PVA hydrogel into two different release mediums, PBS and PBS containing 1 % BSA. As shown, the detected concentration of released Serp-1 was higher for the buffer containing BSA. This effect is due to the presence of BSA which is commonly used as a blocking agent to prevent non-specific interactions between protein and surfaces, thereby ensuring Serp-1 stays in solution after being released from the hydrogel to maximize its detection.



**FIGURE 4.13.** Release profiles of Serp-1 demonstrating the difference in release into different release mediums in the diffusion cell. Shown are a buffer containing 1% BSA (■) and PBS (●).

To confirm the effectiveness of the BSA surface coating, the release profiles of the BSA coated glass diffusion cell and that of the previous study conducted in a polypropylene tube are compared in FIGURE 4.14. It can be seen that the presence of 1 % BSA simultaneously with Serp-1 at low concentrations is effective to prevent any

significant adsorption of Serp-1 on the glass diffusion cell, thus allowing the Serp-1 protein to stay in solution for assay.



**FIGURE 4.14.** Release profiles of Serp-1 in diffusion cells with release mediums of PBS (●) or buffer containing 1% BSA (□), which is compared to Serp-1 release in a polypropylene tube (■).

Following the results of this controlled release experiment, the BSA containing buffer was subsequently used as the release medium for all Serp-1 controlled release studies. This BSA containing buffer was also convenient to use since it is the same buffer used for assay dilutions for the ELISA to detect Serp-1. Therefore, it does not interfere with the detection method and is an appropriate release medium to work with.

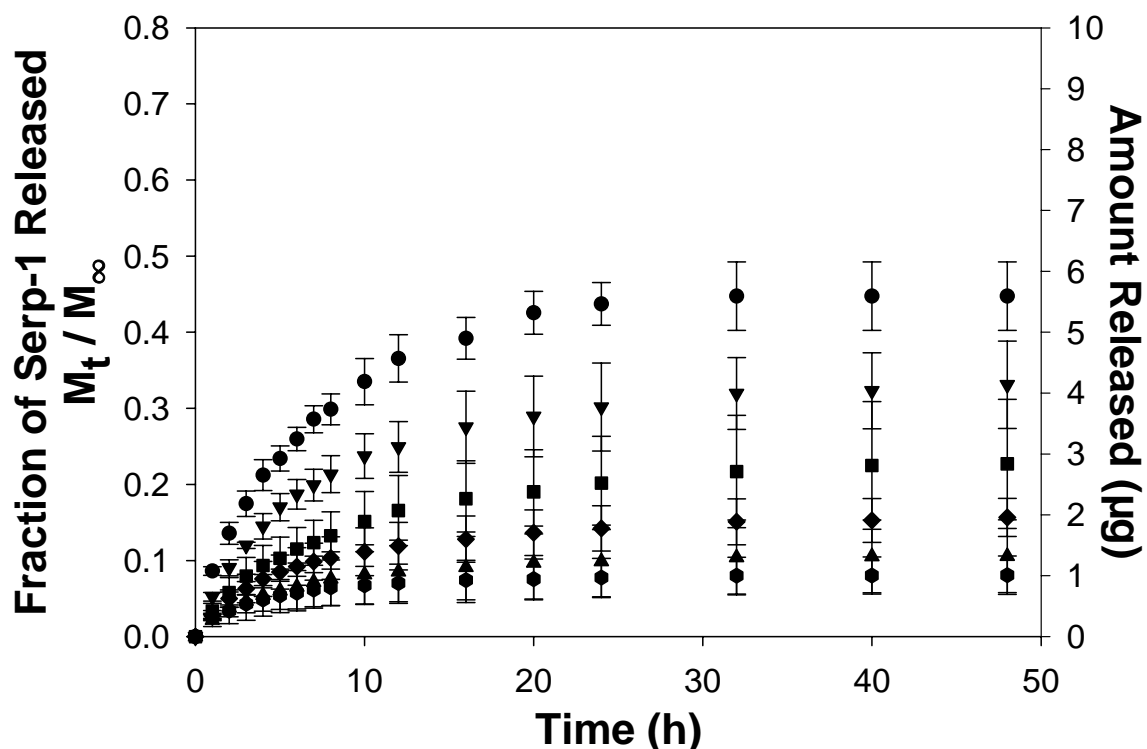
### 4.3 CONTROLLED RELEASE OF SERP-1 FROM PVA HYDROGEL

*In vitro* controlled release experiments were performed using the therapeutic Serp-1 protein to investigate the effect of the key process parameters, including the number of freeze-thaw cycles, PVA solution concentration and freezing and thawing rates, on the release properties of PVA hydrogels. The release kinetics were then analyzed and compared to the model protein, BSA, release characteristics. Finally, in a practical sense, the release was tailored for a therapeutic level of delivery for Serp-1 and the *in vitro* release monitored in human whole blood.

#### 4.3.1 Number of Freeze-Thaw Cycles

One through six freeze-thaw cycles were examined since it is known from a previous study using a similar freeze-thaw protocol that the mechanical properties of the PVA hydrogel reached a steady state after six cycles [11]. The mechanical properties are influenced by the structure of the hydrogel that is formed during the freezing and thawing process as are the controlled release properties, thereby rationalizing the correlation for the number of freeze-thaw cycles. Release experiments were carried out using 10 wt% PVA and 0.001 wt% Serp-1 with constant freezing and thawing rates of 0.1 °C/min from 20 °C to -20 °C. FIGURE 4.15 depicts the results, showing that as the number of cycles was increased the release rate decreased. The PVA hydrogels were able to provide release of Serp-1 for more than 24 hours.

The systematic decrease in release rate is comparable to our model protein studies with BSA and with other groups in literature [13, 135]. Each increasing cycle leads to an increase in the local PVA concentration in the polymer rich regions as the material phase separates, also associated with an increase in the volume fraction of crystalline regions [135]. This effectively decreases the mobility of the protein through the amorphous portions of the polymer rich region giving the decrease in release.



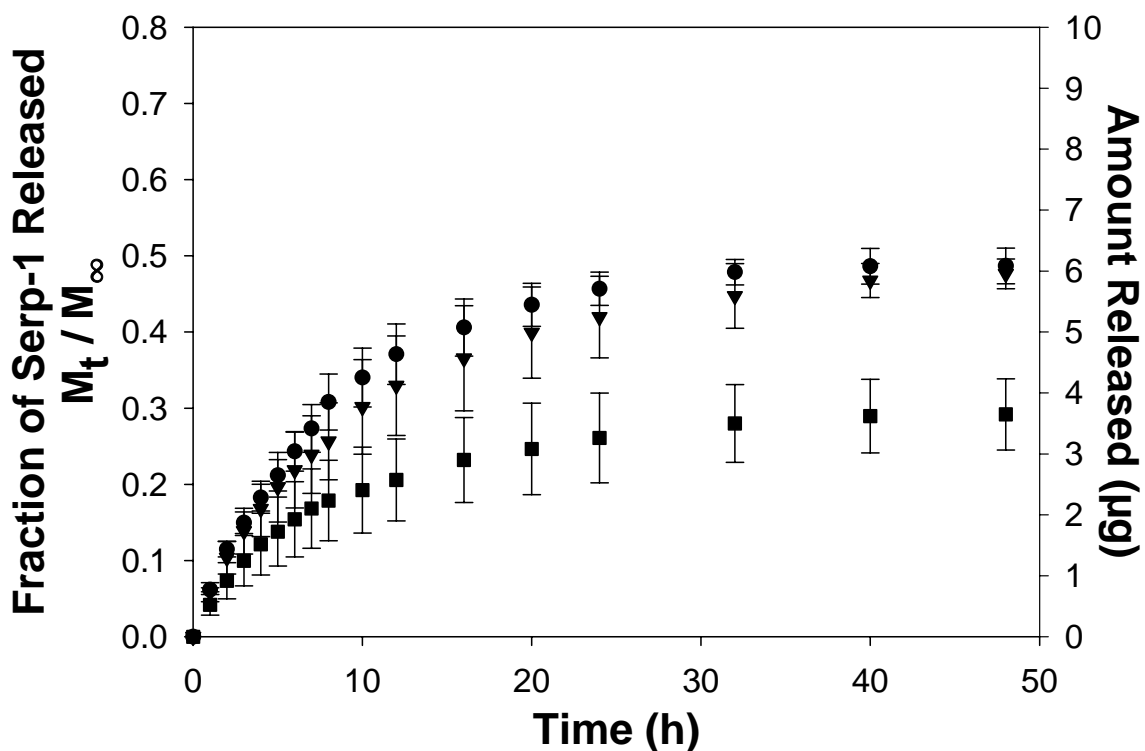
**FIGURE 4.15.** Controlled release profiles demonstrating the effect of the number of freeze-thaw cycles with 1 cycle (●), 2 cycles (▼), 3 cycles (■), 4 cycles (◆), 5 cycles (▲) and 6 cycles (⬤). Studies were performed releasing Serp-1 (0.001 wt%) from PVA (10 wt%) into a buffered medium using Franz diffusion cells at 37 °C. Error bars represent the standard deviation with n=3.

### 4.3.2 PVA Concentration

PVA solution concentrations of 8, 10, and 12 wt% were considered to determine the effect of polymer content on the release. Experiments were performed using two freeze-thaw cycles and 0.001 wt% Serp-1 loading, with constant freezing and thawing rates of 0.1 °C/min from 20 °C to -20 °C. FIGURE 4.16 shows that as the PVA concentration is increased, the release decreases. Samples with 8 wt% and 10 wt% PVA demonstrated close release profiles, while increasing PVA content to 12 wt% did decrease the release rate of Serp-1. The increase in PVA solution concentration results in



an increase in polymer concentration in the polymer rich regions of the hydrogel. This in turn causes a decrease in the mobility of the protein and thus the reduction in diffusion of Serp-1 out of the PVA hydrogel.



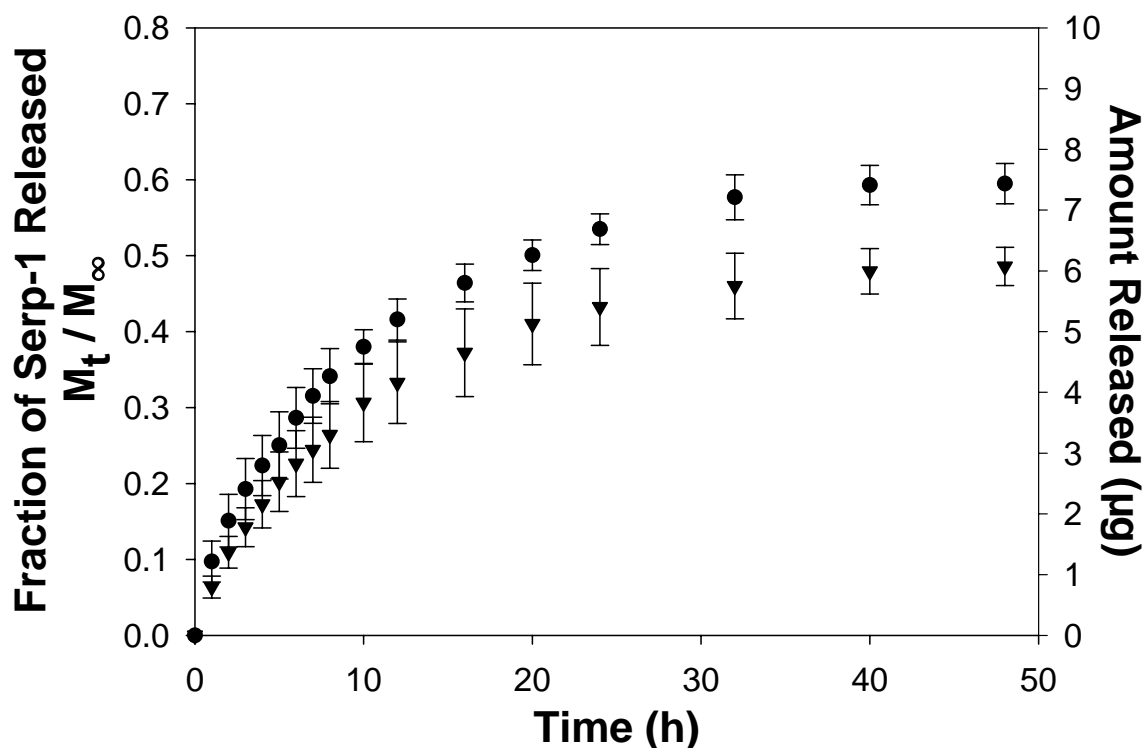
**FIGURE 4.16.** Controlled release profiles demonstrating the effect of concentration of PVA solution with 8 wt% (●), 10 wt% (▼) and 12 wt% (■). Studies were performed releasing Serp-1 (0.001 wt%) from PVA with 2 freeze-thaw cycles into a buffered medium using Franz diffusion cells at 37 °C. Error bars represent the standard deviation with n=3.

### 4.3.3 Freezing Rate and Thawing Rate

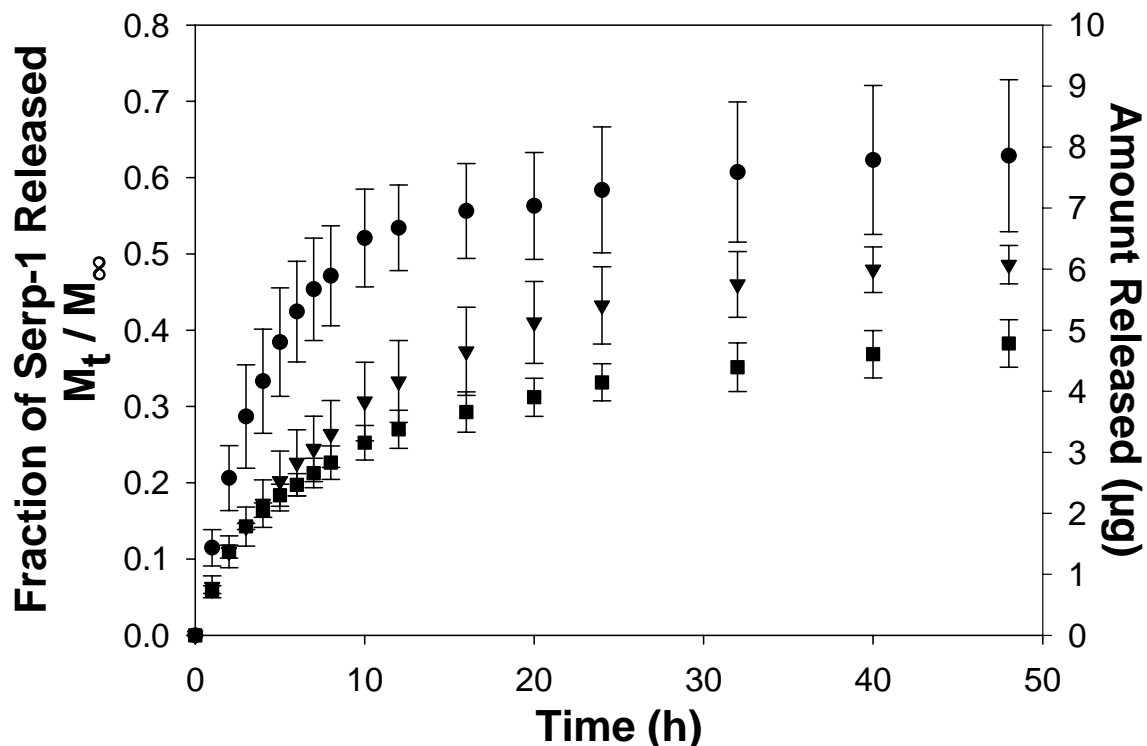
The effect of the freezing rate or thawing rate was investigated. The freezing rate was varied from 0.10 °C/min to 0.15 °C/min while keeping the number of freeze-thaw cycles at two, Serp-1 loading at 0.001 wt%, PVA concentration of 10 wt% and a thawing rate of 0.1 °C/min. FIGURE 4.17 shows that Serp-1 release decreases as the freezing rate

is reduced. Similarly the thawing rate was varied from 0.05 °C/min to 0.10 °C/min to 0.5 °C/min while holding constant the number of freeze-thaw cycles at two, Serp-1 loading at 0.001 wt%, PVA concentration of 10 wt% and a freezing rate of 0.1 °C/min. FIGURE 4.18 demonstrates the effect of increasing the thawing rate leading to an increase in release rate of Serp-1.

A reduction in the freezing or thawing rate creates a longer processing time for the PVA hydrogel. This allows more time for the PVA chains to organize into ordered crystalline domains, resulting in an increase in number and size of the crystallites. This means the density of the polymer rich regions will increase and the mobility of the protein through these regions will be reduced, slowing the release of Serp-1.



**FIGURE 4.17.** Controlled release profiles demonstrating the effect of the freezing rate of the PVA freeze-thaw cycles with 0.15 °C/min (●) and 0.10 °C/min (▼). Studies were performed releasing Serp-1 (0.001 wt%) from PVA (10 wt%) with 2 freeze-thaw cycles into a buffered medium using Franz diffusion cells at 37 °C. Error bars represent the standard deviation with n=3.

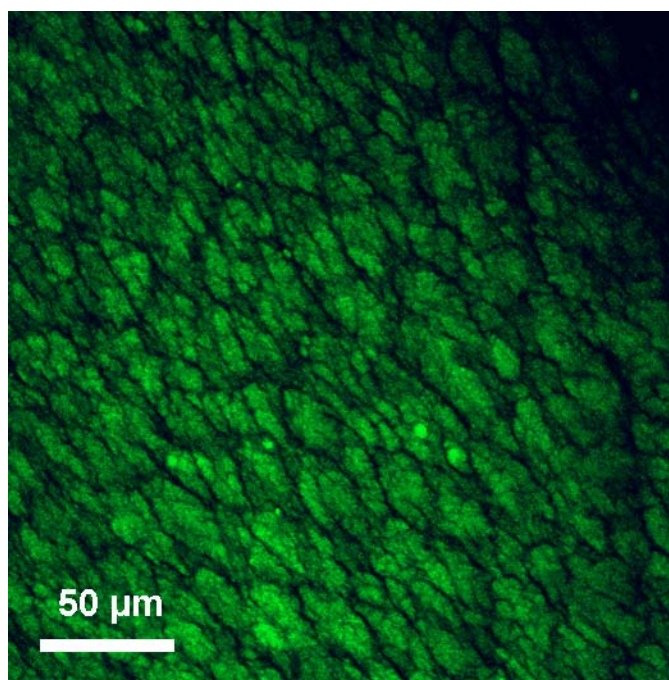


**FIGURE 4.18.** Controlled release profiles demonstrating the effect of the thawing rate of the PVA freeze-thaw cycles with 0.5 °C/min (●), 0.10 °C/min (▼) and 0.05 °C/min (■). Studies were performed releasing Serp-1 (0.001 wt%) from PVA (10 wt%) with 2 freeze-thaw cycles into a buffered medium using Franz diffusion cells at 37 °C. Error bars represent the standard deviation with n=3.

#### 4.3.4 Diffusion Properties of Protein in PVA

Control over the release of protein from PVA hydrogel is largely dependent on the structure of the hydrogel since it is a diffusion controlled release mechanism [13, 153]. The structure of PVA hydrogel arises from phase separation that occurs during the freezing and thawing process. The hydrogel is formed with polymer rich regions and polymer poor regions. The polymer rich regions have a higher local concentration of PVA with ordered domains of crystallites (3 nm) linking the polymer chains and amorphous PVA of dimension 19 nm [145]. The polymer poor regions are greater than

100 nm and pockets of water left behind as the ice crystals melt during the thawing stage [145, 151]. FIGURE 4.19 is an image demonstrating these two regions. A fluorescent probe, fluorescein isothiocyanate conjugated BSA (FITC-BSA), was incorporated into the PVA matrix. The confocal scanning laser microscope (CSLM) image shows the fluorescent protein localized in the polymer poor regions while the PVA rich regions are not fluorescent and are the black zones separating the regions filled with water-soluble protein. This indicates that most of the water-soluble proteins will reside in the water-filled pores when incorporated into the matrix.



**FIGURE 4.19.** CSLM image of FITC-BSA in the PVA matrix. The green represents pockets of FITC-BSA surrounded by the non-fluorescent black of the PVA matrix.

The protein then must diffuse out of the polymer poor regions across the polymer rich regions to be released out of the matrix. For that reason, the structural properties become important, as the space in the polymer mesh allows the migration of protein. The diffusion properties are controlled by controlling the structure from altering the

processing conditions. The results given thus far in this thesis support this phenomenon. As demonstrated, the number of freeze-thaw cycles, PVA concentration, and freezing and thawing rates all affect the diffusion controlled release of protein, which can be used to design a release profile that would be best suited for restenosis control using Serp-1.

Notably, there are some differences when comparing the release profiles of either BSA or Serp-1 from the PVA matrix. Generally, BSA release goes to completion and exhibits close to 100 % release, whereas Serp-1 never reaches 100 %. Of course this is largely dependent on the processing conditions, and when the conditions are such that the release will be slower, the amount released will often be reduced, even with BSA. However, at the faster rates, when BSA demonstrates close to 100 % release, Serp-1 shows only about half of that. Both proteins are of a comparable intermediate size, with BSA at 67 kDa and Serp-1 at 55 kDa. Hence, they should have the ability to migrate through the mesh space in a similar way, and so there must be other reasons for the difference in release properties.

We know from work previously discussed using BSA as a model protein, the ionic strength of the release medium plays a role in the outcome of the amount of protein released. If the ionic strength of the release medium is higher than the solvent used in the PVA hydrogel preparation (90 % water), then an osmotic pressure is induced, drawing water out of the hydrogel. When this happens the structure shrinks and the local PVA concentration increases, contributing to a decrease in observed protein release. The studies done with Serp-1 used a buffer as the release medium, while studies done with BSA used water as the release medium. Therefore, the BSA system would not have induced an osmotic pressure gradient. However, the Serp-1 system likely suffered from the osmotic pressure effect and this is likely part of the reason for the diminished released amount. The reduction due to using 1X PBS for the release medium in the BSA studies was about 30 % (FIGURE 4.8). But, for Serp-1 less than 50 % release is typically observed, so the osmotic pressure is likely not the only contributing factor.

#### 4.3.4.1 Serp-1 Remaining in Matrix

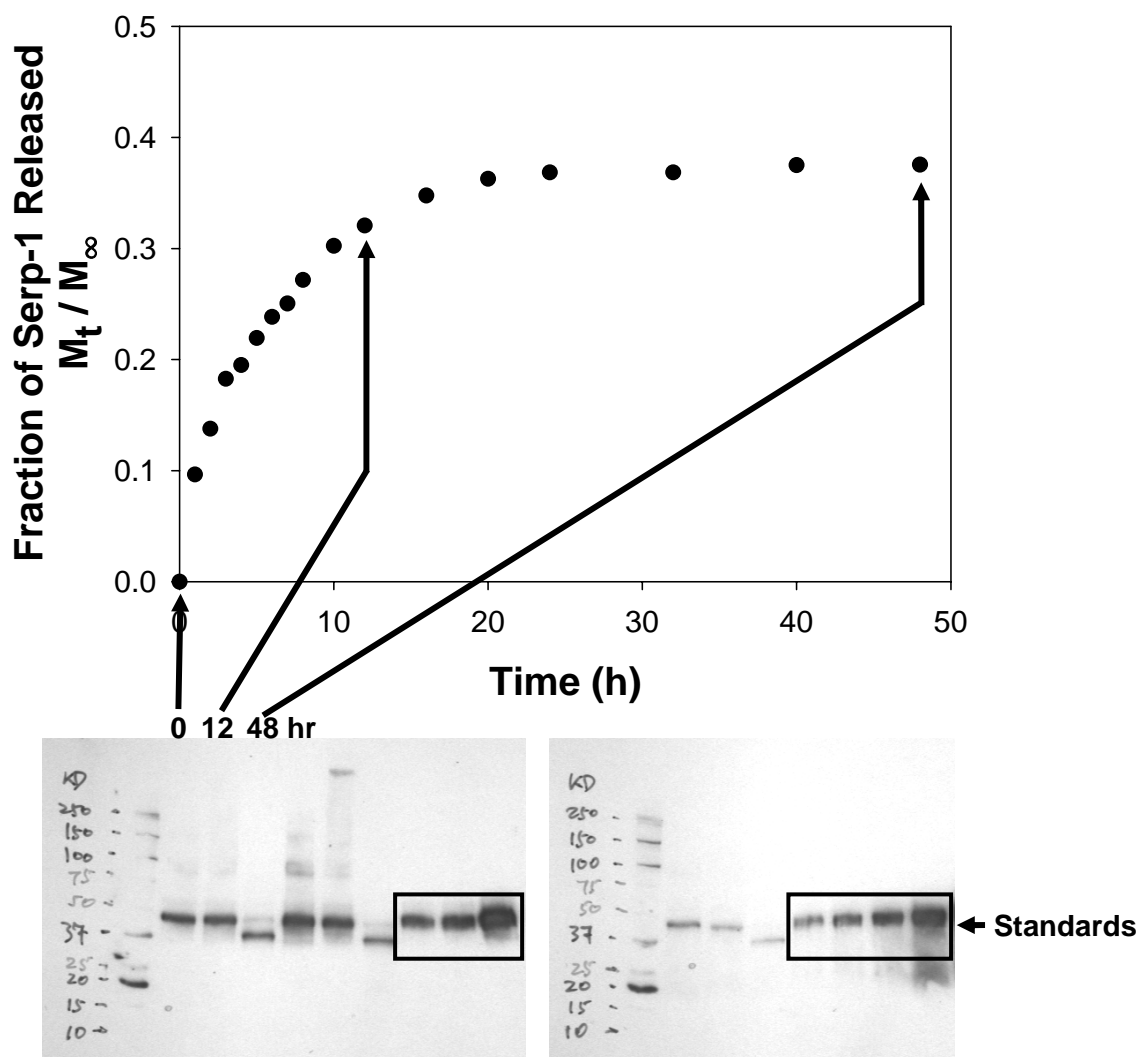
To account for approximately 50 % of Serp-1 not observed on the release profile, there are two possibilities. First, it is possible that a non-active form of Serp-1 is undetected during the assay to determine concentration of Serp-1 released. The concentration is determined by an ELISA using antibodies that are specific only to the active form of Serp-1. If Serp-1, somehow lost activity during the processing and/or release experiment, it would go undetected in the release medium measurements. During processing the protein undergoes multiple freeze-thaw cycles which is a common source of denaturing for many proteins. However, we know that Serp-1 maintains its activity throughout six freeze-thaw cycles as previously shown in FIGURE 4.11.

Unfortunately, there are no antibodies available to detect all forms of Serp-1, so a different approach was required to determine if Serp-1 was only released in the active form and that the remaining protein remained in the PVA matrix. To do this a backward approach was used to measure the amount of Serp-1 remaining in the actual PVA hydrogel following the completion of the release experiment. Release experiments were performed as usual, and three samples were used for different time points. The release profile is given in FIGURE 4.20, showing the three time points considered, 0, 12, and 48 hours. After these time points the PVA hydrogel was collected to determine the amount of Serp-1 remaining in the PVA matrix. The 0 hour sample represents a fully loaded matrix before the release experiment is conducted. The PVA matrix was melted back to liquid form by heating above 70 °C and the liquid samples were tested for Serp-1 content by Western blot. The Western blots (FIGURE 4.20) of the Serp-1 samples were compared to standards and the concentrations of each sample were approximated. These results are shown in TABLE 4.3 and suggest the majority of Serp-1 not released is still within the PVA matrix. Comparing the percentage left in the matrix (TABLE 4.3) to the percentage released (FIGURE 4.20), the two amounts add up to approximately 100 % for all three time points.

There is a possibility that Serp-1 interacts more strongly with the PVA matrix than BSA. If this is the case, there is the potential that a significant amount of Serp-1 is

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simply trapped in the PVA matrix and cannot be released. This is confirmed by these results demonstrating a large amount of Serp-1 remains in the PVA matrix after the release experiment is completed. For clinical applications, the interaction between Serp-1 and the PVA matrix and its effects on the controlled release of Serp-1 must be considered. It is clear that a higher initial loading will be required. The initial loading must take into account that a portion of Serp-1 will remain in the PVA matrix and will not be released to be of therapeutic use. The processing conditions of the PVA system will likely affect the percentage of Serp-1 trapped since these conditions influence the structure of the hydrogel. A systematic study could be done to determine the percentage of Serp-1 remaining in the PVA matrix for all the different processing conditions discussed in previous sections. With that knowledge, the required increase in initial loading could be determined and accounted for when designing a controlled release PVA/Serp-1 system for specific clinical applications.



**FIGURE 4.20.** Determination of Serp-1 content remaining in the PVA matrix after release reaches completion. The top shows the release profile of Serp-1 from PVA hydrogel. The bottom shows Western blots of the PVA matrix after 0, 12 and 48 hours. By comparing to the standards, the approximately concentration of each sample can be determined.



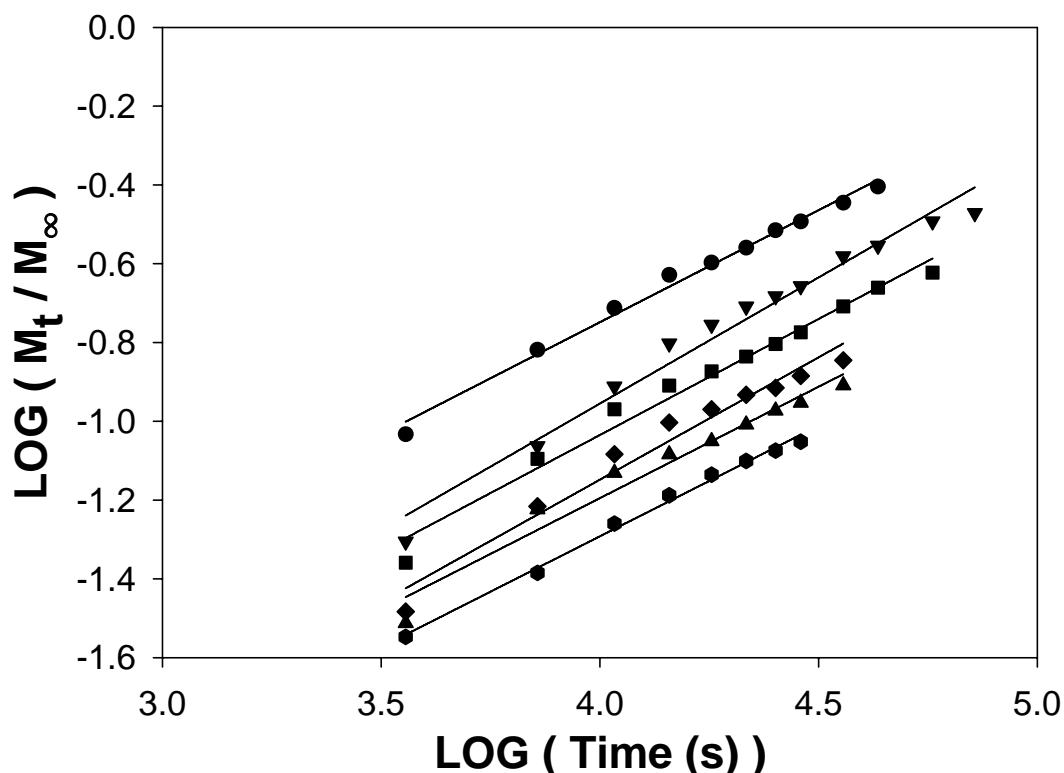
**TABLE 4.3.** Concentration of Serp-1 remaining in the PVA matrix following release after 0, 12, and 48 hours as calculated from the Western blots. The concentration is converted into percentage to compare to the amount released from the release profile.

	<b>Approximated Concentration</b>	<b>Percentage Left in Matrix</b>
0 hr	0.34 mg/mL	100 %
12 hr	0.24 mg/mL	69 %
48 hr	0.13 mg/mL	49 %

### 4.3.5 Release Kinetics of Serp-1

Thus far, the data has been represented in release profiles plotted as the cumulative fractional protein release ( $M_t/M_\infty$ ) versus time. Each study was done in triplicate and the average curve was plotted with error bars representing the standard deviation. These curves are now further analyzed using well known models to quantify the release kinetics and distinguish the effect of the different processing parameters.

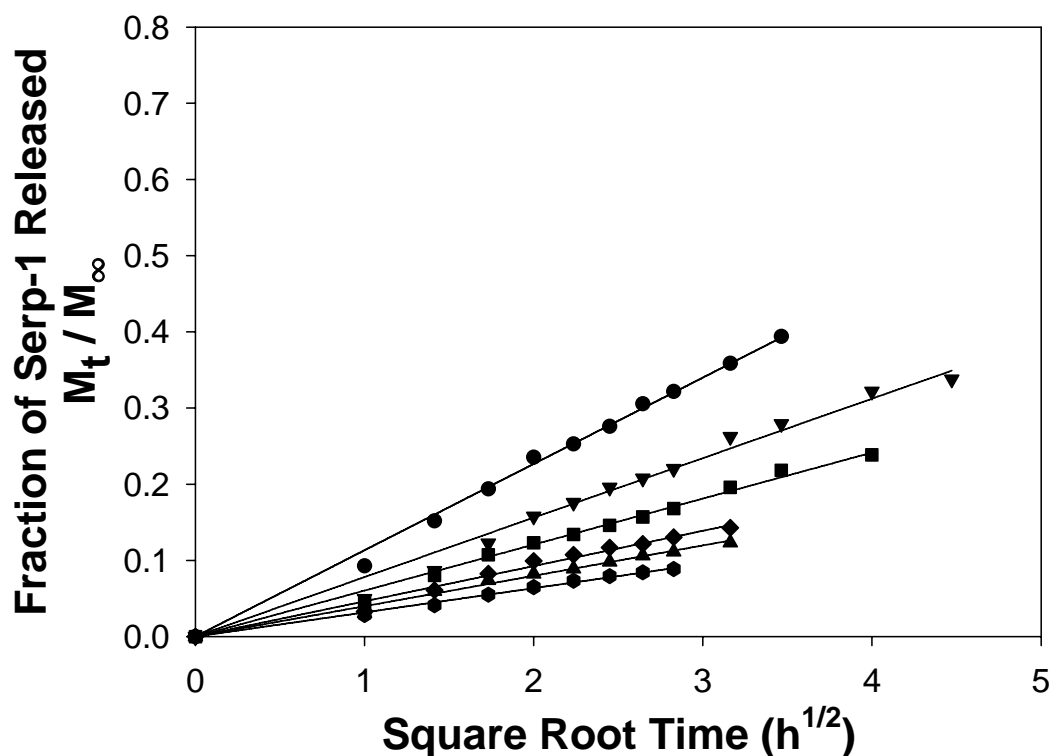
Protein release from PVA has previously been classified as a diffusion controlled mechanism [13, 153]. To verify this, the Power Law (EQUATION 2.5) was initially used to classify the release behaviour of our data. This relationship is valid for the first 60 % of release and for a thin slab a release exponent,  $n$ , value of 0.5 represents Fickian diffusion [92]. The release profiles were converted to log-log plots to determine the value of  $n$ , which corresponds to the slope. Linear regression was used to find the slope of each log-log plot. FIGURE 4.21 shows a typical log-log plot for Serp-1 release as a function of the number of freeze-thaw cycles.



**FIGURE 4.21.** An example of using the Power Law to find the release exponent,  $n$ . Shown here is a log-log plot of the release profile of Serp-1 from PVA as a function of the number of freeze-thaw cycles, with 1 cycle (●), 2 cycles (▼), 3 cycles (■), 4 cycles (◆), 5 cycles (▲) and 6 cycles (⬢). The slope of log-log plot is representative of  $n$ .  $R^2$  values for the linear fits are 0.9910 (cycle 1), 0.9763 (cycle 2), 0.9795 (cycle 3), 0.9659 (cycle 4), 0.9601 (cycle 5), and 0.9943 (cycle 6).

The results are summarized in TABLE 4.4. The values of  $n$  ranged from 0.44 to 0.67 which means they were close to 0.5 and thus it can be considered to be diffusion controlled release. It should also be noted that a value of  $n$  between 0.5 and 1.0 specifies anomalous transport with a contribution from chain relaxation as well as diffusion [166]. Some of the conditions tested would result in less physical crosslinking than others and this would affect the release exponent value. For example, those samples with the fastest thawing rate or with the lowest PVA concentration had the highest release exponent suggesting they may have had additional relaxation or swelling contributing to a higher  $n$  value.

Since most release exponent values were close to 0.5, the Diffusion Model applies. This model has two fits for the early and late time approximations as represented by EQUATION 2.8 and EQUATION 2.9. It is not uncommon to use only the first equation for analysis of the first 60 % of release. The early time approximation states that  $M_t/M_\infty$  is proportional to the square root of time. Therefore, a plot of  $M_t/M_\infty$  versus the square root of time was used to determine the slope which represents  $4^2(D/\pi l^2)$ . FIGURE 4.22 shows a square root of time plot for Serp-1 release as a function of the number of freeze-thaw cycles. From the slope, the value of the diffusion coefficient was found and is given in TABLE 4.4.



**FIGURE 4.22.** An example of using the square root of time versus  $M_t/M_\infty$  to find the diffusion coefficient,  $D$ . Shown here is the release profile of Serp-1 from PVA as a function of the number of freeze-thaw cycles, with 1 cycle ( $\bullet$ ), 2 cycles ( $\blacktriangledown$ ), 3 cycles ( $\blacksquare$ ), 4 cycles ( $\blacklozenge$ ), 5 cycles ( $\blacktriangle$ ) and 6 cycles ( $\blacklozenge$ ). The slope of representative of  $4^2(D/\pi l^2)$  from the early time approximation of the Diffusion Model.  $R^2$  values for the linear fits are 0.9956 (cycle 1), 0.9832 (cycle 2), 0.9916 (cycle 3), 0.9845 (cycle 4), 0.9896 (cycle 5), and 0.9949 (cycle 6).

**TABLE 4.4.** Controlled release of Serp-1 from PVA with varying PVA processing parameters. Shown are the release exponent values from the Ritger-Peppas model and diffusion coefficients for each combination of PVA processing parameters studied.

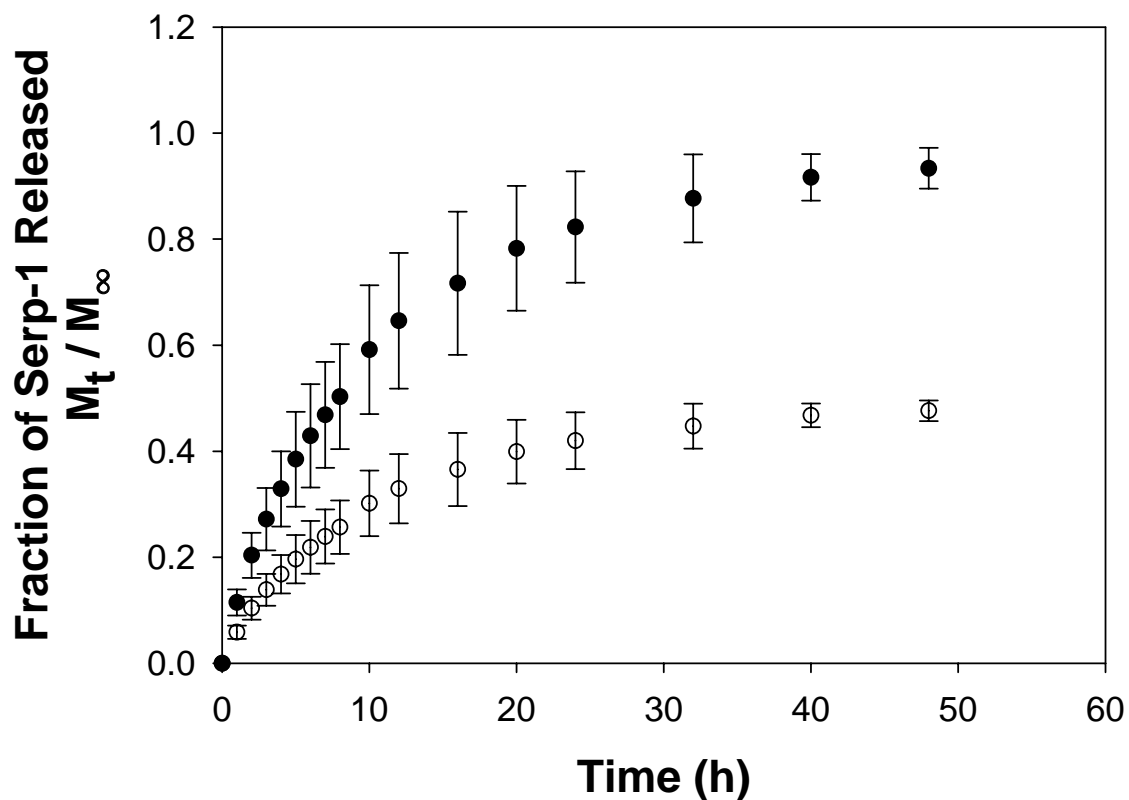
Matrix Parameters				Release Exponent	Apparent Diffusion Coefficient
Number of Freeze-Thaw Cycles	PVA Concentration (%)	Freezing Rate (°C/min)	Thawing Rate (°C/min)	$n \pm$ Standard Deviation	$D_{app} \pm$ Standard Deviation ( $\times 10^{-9}$ cm <sup>2</sup> /s)
1	10	0.10	0.10	$0.57 \pm 0.05$	$12.8 \pm 7.9$
2	10	0.10	0.10	$0.63 \pm 0.01$	$9.2 \pm 4.1$
3	10	0.10	0.10	$0.62 \pm 0.03$	$4.6 \pm 4.4$
4	10	0.10	0.10	$0.60 \pm 0.02$	$2.7 \pm 2.4$
5	10	0.10	0.10	$0.56 \pm 0.03$	$1.8 \pm 2.3$
6	10	0.10	0.10	$0.44 \pm 0.12$	$1.2 \pm 0.9$
2	8	0.10	0.10	$0.66 \pm 0.07$	$13.0 \pm 1.0$
2	10	0.10	0.10	$0.62 \pm 0.02$	$10.4 \pm 2.8$
2	12	0.10	0.10	$0.63 \pm 0.04$	$5.3 \pm 3.5$
2	10	0.15	0.10	$0.55 \pm 0.08$	$17.2 \pm 2.3$
2	10	0.10	0.05	$0.58 \pm 0.04$	$7.1 \pm 1.1$
2	10	0.10	0.50	$0.67 \pm 0.03$	$35.9 \pm 10.3$

The apparent diffusion coefficients determined by this method ranged from 1.2 to  $35.9 \times 10^{-9} \text{ cm}^2/\text{s}$  showing a range of control for the various parameters tested. The standard deviations of the apparent diffusion coefficients and release exponents are also given in TABLE 4.4. The standard deviations show the variability within a sample size of three. The standard deviation values for the release exponents are low and demonstrate there is a reasonable amount of variability around the mean. The standard deviation values for the apparent diffusion coefficients, however, demonstrate variability. This is likely due to experimental error and batch to batch inconsistency during sample preparation. This could be further improved upon by repeating the experiments more than three times which may narrow the standard deviation.

In comparison to the apparent diffusion coefficients in TABLE 4.4, the diffusion coefficients found for BSA were on the order of  $10^{-8} \text{ cm}^2/\text{s}$ , as were those from previous work of Yang [12]. Although the proteins are of comparable size Serp-1 appears to release at a slower rate. Since the release rate of a protein from a hydrogel matrix is also dependent on its interaction with the matrix [96], it is therefore possible that Serp-1 interacts more strongly with PVA hydrogels than BSA. From the results shown in the previous section 4.3.4.1, we have demonstrated that a portion of the Serp-1 has been permanently trapped in the PVA matrix and none of the conditions tested led to 100% release. The identification of trapped Serp-1 is very important in the interpretation of the controlled release data. In the Diffusion Model, the determination of  $D$  requires a knowledge of the initial protein loading,  $M_\infty$ . If part of the initially loaded Serp-1 is trapped, then  $M_\infty$  would have to be adjusted accordingly. The values of diffusion coefficients in TABLE 4.4 were calculated without taking into account the trapped Serp-1 and therefore they do not represent the true diffusion coefficients and are therefore termed  $D_{app}$ .

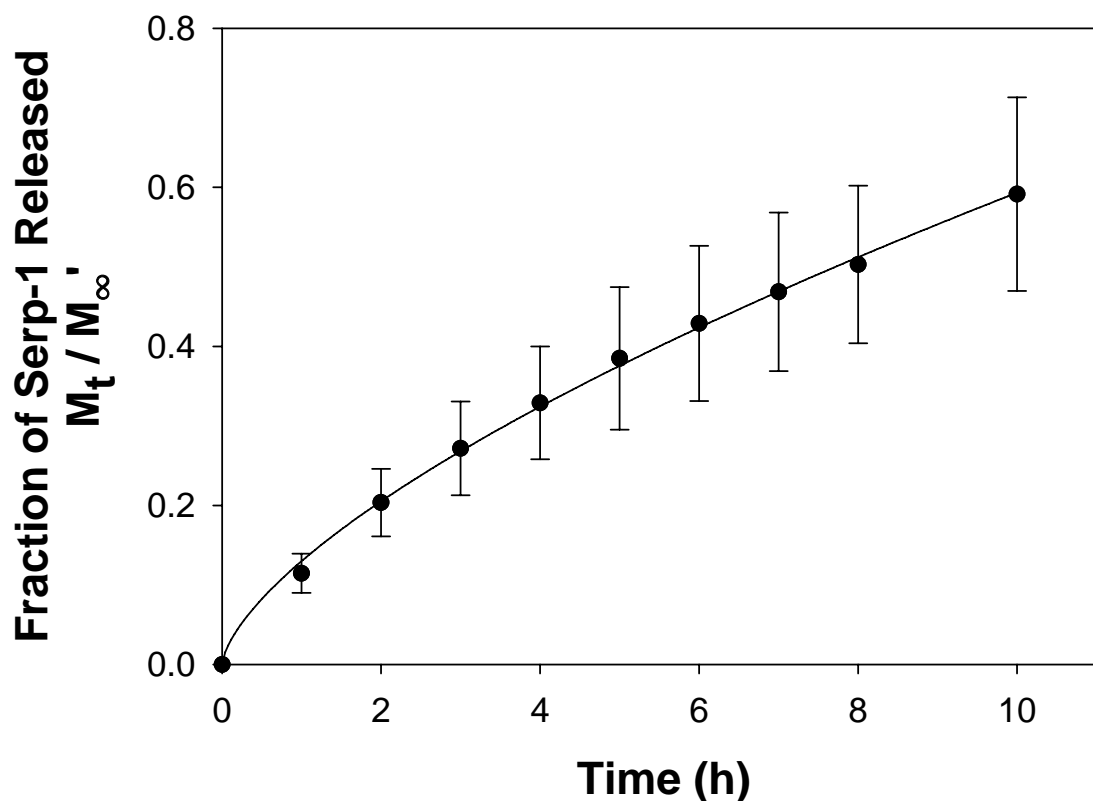
Knowing that some Serp-1 remains in the PVA matrix following the release studies and from TABLE 4.3 that 49 % Serp-1 remains after the release process is completed for a sample of two freeze-thaw cycles, it is possible to re-calculate the diffusion coefficient,  $D$ . This was done by re-plotting the release profile by using a  $M_\infty$  value that takes into account the portion of Serp-1 that was not released. The 49 %

remaining was determined for a sample prepared with 10 wt% PVA, two freeze-thaw cycles and freezing and thawing rates of 0.1 °C/min. The percentage of Serp-1 remaining in the matrix will likely vary when the processing parameters are changed due to structural changes in the hydrogel that may trap more or less protein. Thus, data with the same processing conditions was used when re-plotting to account for the 49 % Serp-1 remaining in the PVA matrix. Data was corrected by using a corrected  $M_\infty$  value,  $M_\infty'$ , equivalent to  $0.51 \cdot M_\infty$ . FIGURE 4.23 displays the original release profile and the corrected profile. By taking into account 49 % of the original drug loading remains in the matrix, the release profile now represents the true release profile.

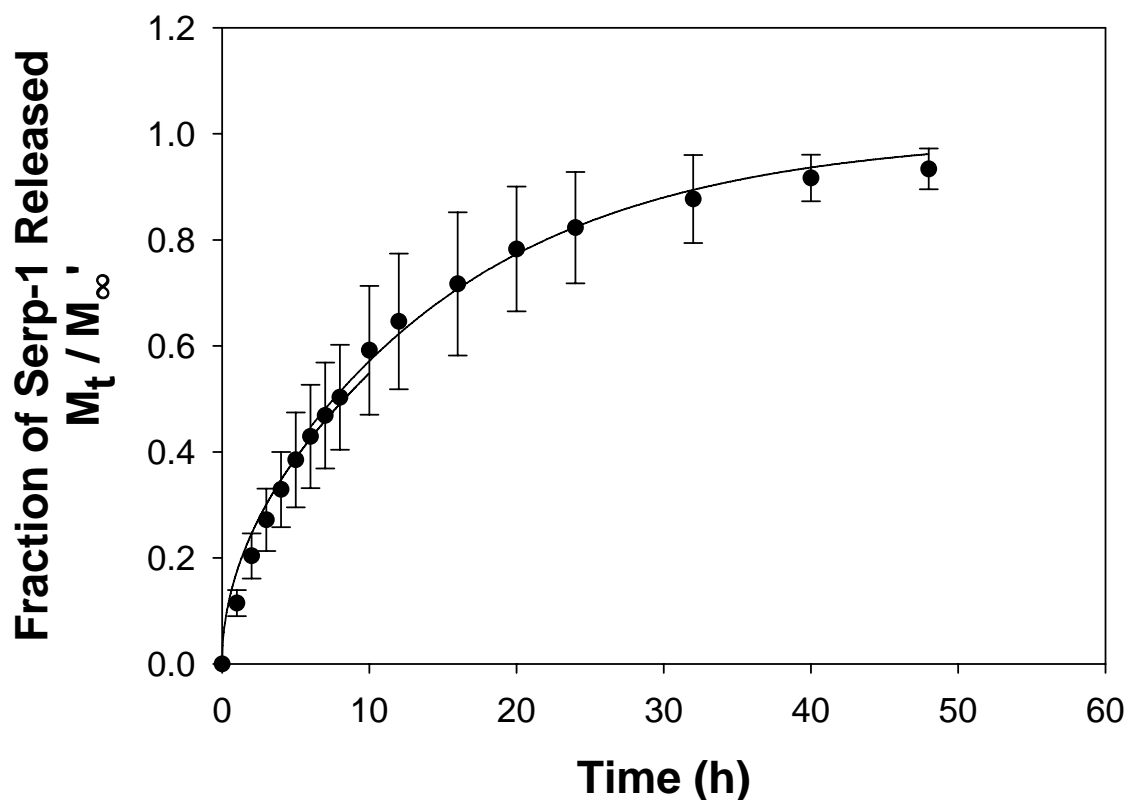


**FIGURE 4.23.** Controlled release profile of Serp-1 from 10 wt% PVA with 2 freeze-thaw cycles from original data (○) and the corrected  $M_\infty' = 0.51 \cdot M_\infty$  (●) to account for 49 % of Serp-1 remaining in the PVA matrix. Error bars represent the standard deviation with  $n=3$ .

The corrected release profile was fitted with the Power Law (EQUATION 2.5) and the Diffusion Model (EQUATION 2.8 and EQUATION 2.9). FIGURE 4.24 shows the Power Law fit and FIGURE 4.25 shows the Diffusion Model fits for the early and late time approximations. Both models fit the data well. A summary of the results of this correction is given in TABLE 4.5 along with the uncorrected values calculated for  $D$  and  $n$ .



**FIGURE 4.24.** Power Law fit of the controlled release profile of Serp-1 from 10 wt% PVA with 2 freeze-thaw cycles corrected with  $M_\infty' = 0.51 \cdot M_\infty$  to account for 49 % of Serp-1 remaining in the PVA matrix.  $R^2 = 0.9984$ . Error bars represent the standard deviation with  $n=3$ .



**FIGURE 4.25.** Diffusion Model fit of the controlled release profile of Serp-1 from 10 wt% PVA with 2 freeze-thaw cycles corrected with  $M_\infty' = 0.51 \cdot M_\infty$  to account for 49 % of Serp-1 remaining in the PVA matrix. Shown are the early-time approximation ( $R^2 = 0.9722$ ) and late-time approximation ( $R^2 = 0.9905$ ). Error bars represent the standard deviation with  $n=3$ .

**TABLE 4.5.** Release exponents and diffusion coefficients of the uncorrected data and corrected data to account for 49 % of Serp-1 remaining in the PVA matrix for Serp-1 release from 10 wt% PVA with 2 freeze-thaw cycles.

	Release Exponent, $n$	Diffusion Coefficient, $D_{early}$ ( $\text{cm}^2/\text{s}$ )	Diffusion Coefficient, $D_{late}$ ( $\text{cm}^2/\text{s}$ )
Uncorrected Data (from TABLE 4.4)	0.62	$10.4 \times 10^{-9}$	n/a
Corrected Data (from FIGURE 4.24 and FIGURE 4.25)	0.66	$3.86 \times 10^{-8}$	$4.22 \times 10^{-8}$



From TABLE 4.5 it can be seen that the two diffusion coefficients of the original uncorrected data and the corrected data do not match. The original plot gives a diffusion coefficient that is underestimated by a factor close to 4. The corrected  $M_\infty$  gives a good fit to the Diffusion Model. The new corrected diffusion coefficient is on the order of  $10^{-8}$   $\text{cm}^2/\text{s}$ , which is consistent with the previously determined results for BSA release from PVA hydrogel [12]. Therefore, the apparent diffusion coefficients shown in TABLE 4.4 are not the actual diffusion coefficients unless they are corrected by taking into account the amounts of Serp-1 trapped in the hydrogel matrix.

It is interesting to note that although the diffusion coefficient is dependent on the value of  $M_\infty$ , the release exponent,  $n$ , as defined in EQUATION 2.5 is not. This is confirmed by the values of  $n$  determined in TABLE 4.5. Correcting the release data to account for the portion of trapped Serp-1 will still result in the same slope for the log-log plot used to determine  $n$ . Therefore, the values of  $n$  in TABLE 4.4 are still applicable and are indicative of diffusion controlled release.

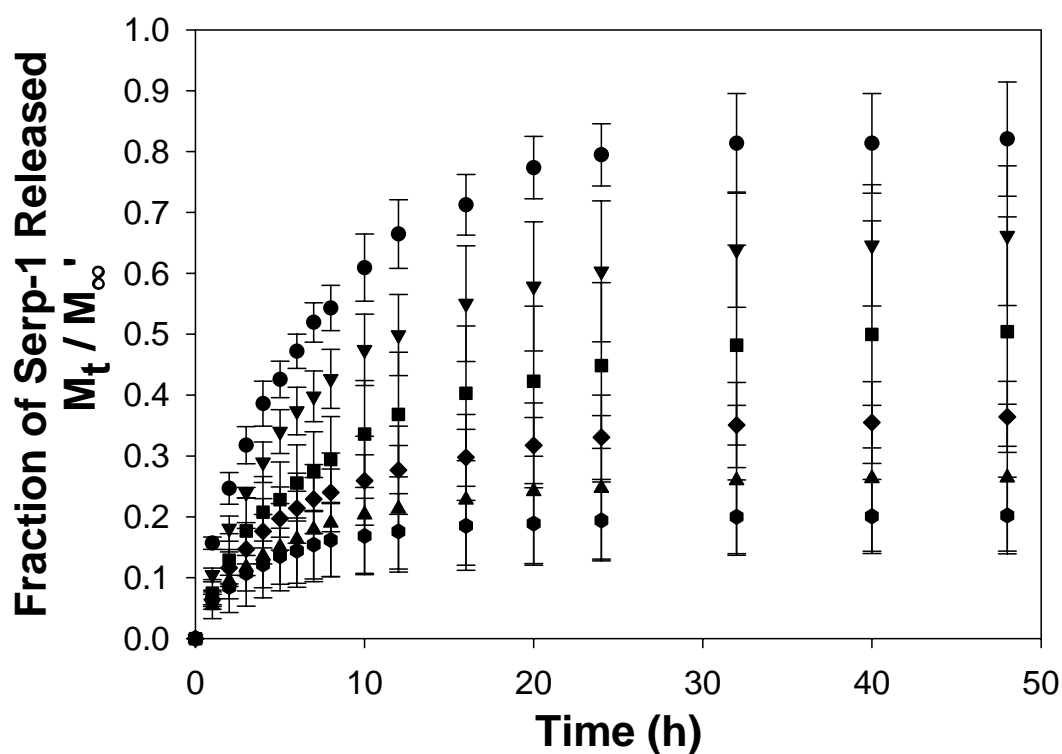
To determine the correct diffusion coefficients, the data sets for all the conditions tested need to be corrected to account for the portion of Serp-1 trapped irreversibly in the matrix. However, this percentage will vary with the processing conditions used. The PVA hydrogel processing conditions have an effect on the resulting hydrogel structure which in turn influences the release properties. It is expected that an increase in the number of freeze-thaw cycles would result in an increase in the amount of Serp-1 being trapped. For instance, PVA hydrogel of six freeze-thaw cycles will likely trap more Serp-1 than one prepared with two freeze-thaw cycles. Structurally this can be understood in terms of an increase in the volume fraction of the crystalline region with increasing number of freeze-thaw cycles [135]. Accordingly, the experiment conducted in section 4.3.4.1 to determine the amount of Serp-1 remaining in the PVA matrix following the release study, needs to be repeated for all the conditions tested. With that information, the entire data set could then be corrected to account for the portion of Serp-1 remaining in the matrix. The Diffusion Model could then be applied to determine the diffusion coefficients correctly.

Due to a variety of reasons, other than for two freeze-thaw cycles, we were not able to determine the amount of Serp-1 trapped in the PVA matrix. In order to arrive at the diffusion coefficients as a function of the number of freeze-thaw cycles, we made reasoning by drawing a parallel to the tensile mechanical properties of PVA. It is well known that the stiffness of this type of PVA hydrogel increases with increasing number of freeze-thaw cycles but this increase gradually tapers off in a non-linear way up to 10 freeze-thaw cycles [11, 167]. It is also known that this increase in the hydrogel stiffness is related to an increase in the volume fraction of crystalline regions of the hydrogel [144, 145]. Since any Serp-1 trapped is in the crystalline regions, it can be reasoned that the amount of the protein trapped is directly proportional to the increase in the PVA hydrogel crystalline regions as expressed in terms of increasing stiffness. Based on this argument and data in the literature [11], together with the data determined for the two freeze-thaw cycles sample presented in section 4.3.4.1, estimates given in TABLE 4.6 can be made.

**TABLE 4.6.** Calculated values for corrected  $M_{\infty}$  to account for the trapped Serp-1 as a function of the number of freeze-thaw cycles.

<b>Number of Freeze-Thaw Cycles</b>	<b>Serp-1 Trapped</b>	<b>Calculated <math>M_{\infty}</math> ' </b>
1	0.45	$0.55 \cdot M_{\infty}$
2	0.50	$0.50 \cdot M_{\infty}$
3	0.55	$0.45 \cdot M_{\infty}$
4	0.58	$0.43 \cdot M_{\infty}$
5	0.60	$0.40 \cdot M_{\infty}$
6	0.60	$0.40 \cdot M_{\infty}$

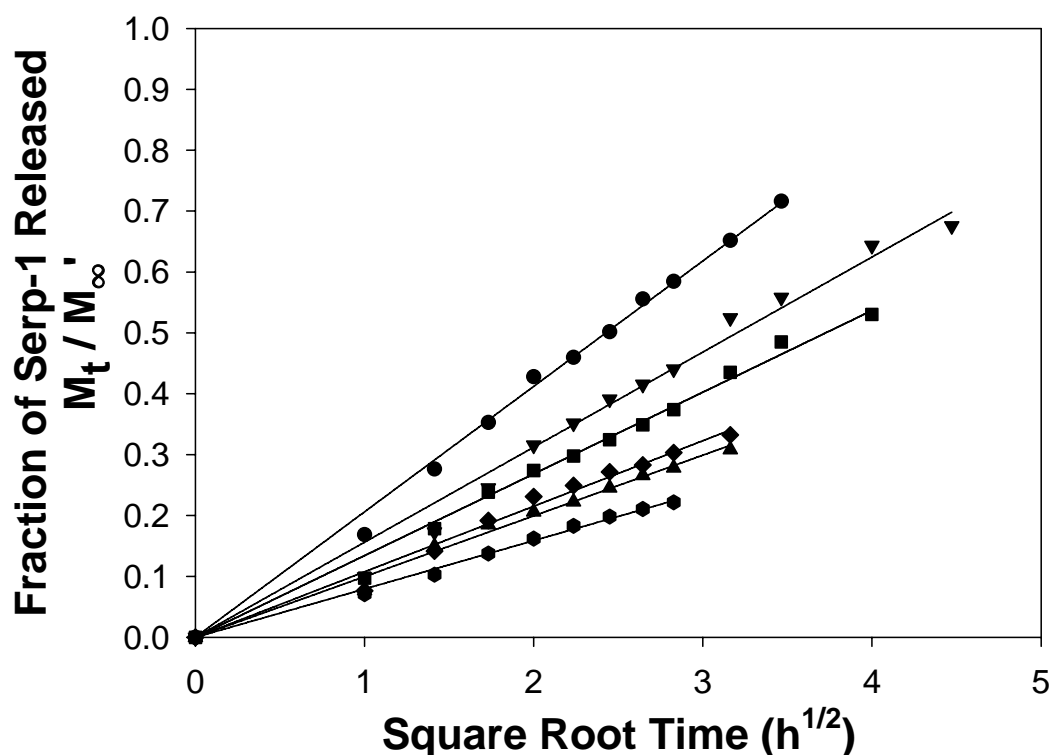
Based on the calculated  $M_\infty$  or  $M_\infty'$ , the experimental release data as a function of the number of freeze-thaw cycles shown in FIGURE 4.15 were re-calculated and are presented in FIGURE 4.26.



**FIGURE 4.26.** Release profiles corrected using  $M_\infty'$  to account for Serp-1 trapped in PVA matrix. The effect of the number of freeze-thaw cycles is demonstrated with 1 cycle (●), 2 cycles (▼), 3 cycles (■), 4 cycles (◆), 5 cycles (▲) and 6 cycles (⬢). Studies were performed releasing Serp-1 (0.001 wt%) from PVA (10 wt%) into a buffered medium using Franz diffusion cells at 37 °C. Error bars represent the standard deviation with n=3.

The diffusion coefficients can now be calculated using the corrected data by way of a  $M_t/M_\infty'$  versus the square root of time plot, as shown in FIGURE 4.27. The diffusion coefficients obtained are collected in TABLE 4.7. It can be seen that the calculated diffusion coefficients are on the order of  $10^{-8}$  cm<sup>2</sup>/s and reflect more closely a range

which is similar to the results from the BSA release studies. The trend also demonstrates a decreasing diffusion coefficient with increasing number of freeze-thaw cycles which correlates to a similar trend of increasing stiffness reported for tensile mechanical properties of the PVA hydrogel [11]. This illustrates the importance of accounting for any portion of the initial loading that may not be released and correcting  $M_\infty$  accordingly for release kinetics determination.



**FIGURE 4.27.** An example of using the square root of time versus  $M_t/M_\infty$  to find the diffusion coefficient,  $D$ . Shown here are the release profiles of Serp-1 corrected by  $M_\infty$  to account for Serp-1 trapped in PVA matrix. The effect of the number of freeze-thaw cycles is demonstrated, with 1 cycle (●), 2 cycles (▼), 3 cycles (■), 4 cycles (◆), 5 cycles (▲) and 6 cycles (●). The slope of representative of  $4^2(D/\pi l^2)$  from the early time approximation of the Diffusion Model.  $R^2$  values for the linear fits are 0.9956 (cycle 1), 0.9832 (cycle 2), 0.9916 (cycle 3), 0.9845 (cycle 4), 0.9896 (cycle 5), and 0.9949 (cycle 6).

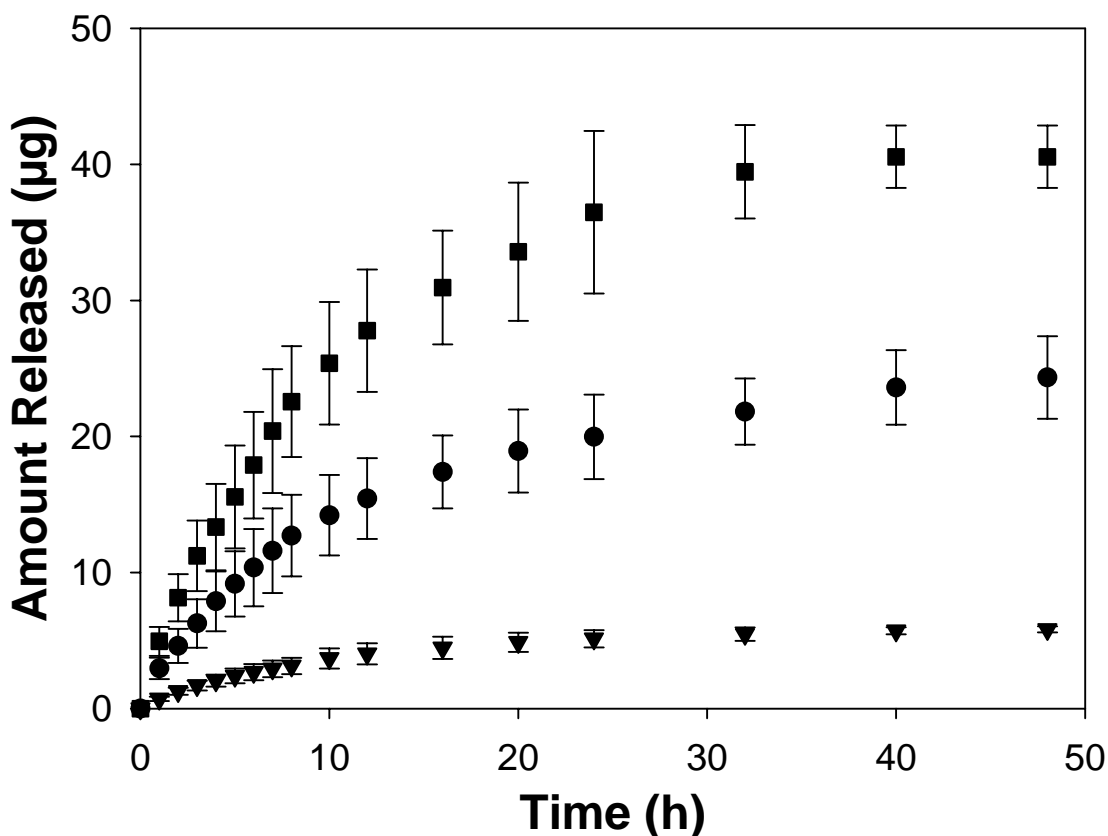
**TABLE 4.7.** Controlled release of Serp-1 from PVA as a function of the number of freeze-thaw cycles. Shown are the diffusion coefficients for the corrected data set using calculated  $M_{\infty}'$  values to account for a portion of Serp-1 remaining in the PVA matrix. Studies were performed releasing Serp-1 (0.001 wt%) from PVA (10 wt%) processed with freezing and thawing rates of 0.10 °C/min.

Number of Freeze-Thaw Cycles	Calculated $M_{\infty}'$	Diffusion Coefficient
		$D \pm$ Standard Deviation ( $\times 10^{-8}$ cm <sup>2</sup> /s)
1	$0.55 \cdot M_{\infty}$	$4.2 \pm 2.5$
2	$0.50 \cdot M_{\infty}$	$3.7 \pm 1.6$
3	$0.45 \cdot M_{\infty}$	$2.3 \pm 2.1$
4	$0.43 \cdot M_{\infty}$	$1.4 \pm 1.2$
5	$0.40 \cdot M_{\infty}$	$1.1 \pm 1.3$
6	$0.40 \cdot M_{\infty}$	$0.8 \pm 0.6$

#### 4.3.6 Protein Loading

Protein loading was also investigated to determine its effect on the release profile of Serp-1 from PVA hydrogel. In FIGURE 4.28, it can be seen that by using loadings of 0.001 wt%, 0.005 wt%, and 0.010 wt%, the amount of Serp-1 released increased with increased loading. Again, for this study all other parameters were held constant with the number of freeze-thaw cycles at two, PVA concentration of 10 wt% and freezing and thawing rates of 0.1 °C/min.

The results of changing the protein loading are not an effect of the processing parameters like the aforementioned studies, but merely provide a simple manner to modify the release amount of Serp-1. The release kinetics should be quite similar for each loading, only the amount released will vary. The Serp-1 loadings studied here are below the therapeutic level, however suggest by increasing the loading an appropriate dosage may be reached. In the next section, more therapeutically relevant loadings are tested.



**FIGURE 4.28.** Controlled release profiles demonstrating the effect of the drug loading with 0.010 wt% Serp-1 (■), 0.005 wt% Serp-1 (●), and 0.001 wt% Serp-1 (▼). Studies were performed releasing Serp-1 from PVA (10 wt%) with 2 freeze-thaw cycles into a buffered medium using Franz diffusion cells at 37 °C. Error bars represent the standard deviation with n=3.

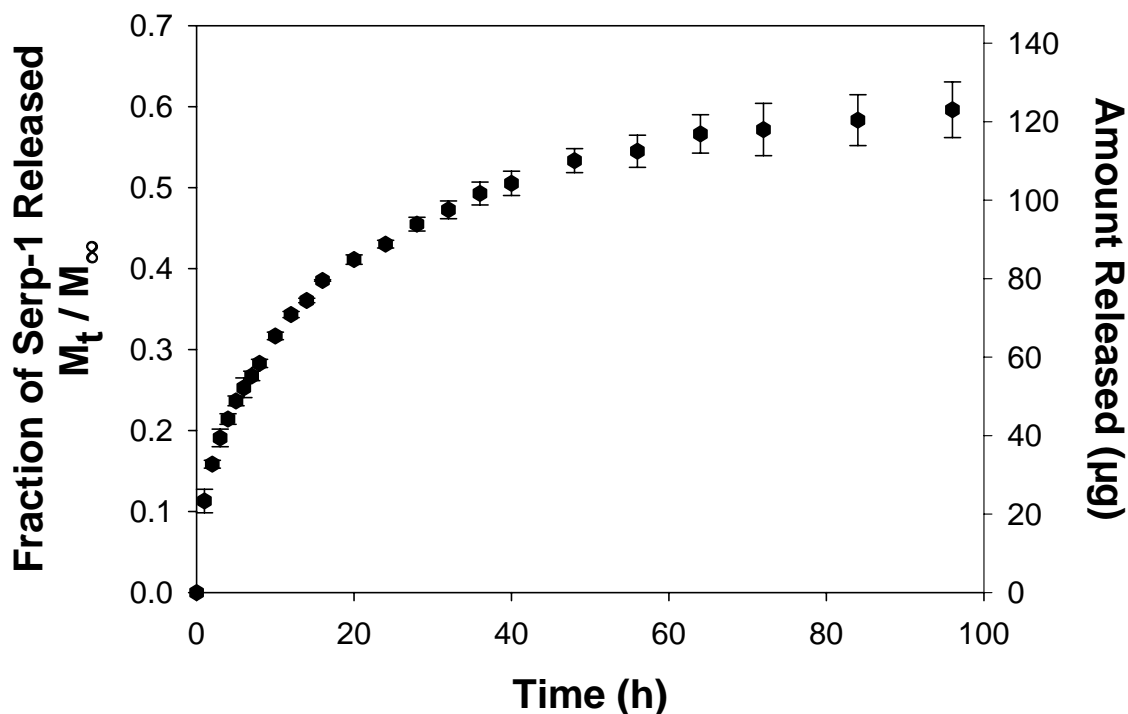
#### 4.3.7 Therapeutic Dosage and Timeframe

For a more practical approach, a higher loading is required for a therapeutic level of delivery to be achieved. *In vitro* release experiments were conducted to establish if a therapeutic level of Serp-1 could be loaded into the PVA matrix and released in an appropriate timeframe. Since Serp-1 is still under investigation in clinical trials, it has yet to be decided what release profile would be satisfactory. For an estimated timeframe it is thought that if the delivery can be sustained for more than the half-life of injected Serp-1,

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approximately one hour, the system would be successful. A typical dose for animal experiments was used as a goal to determine an appropriate dosage to aim for. Rat experiments with Serp-1 use 0.5 mg/kg of rat body mass, and an average rat weighs 250 g which works out to be an average dose of 125  $\mu$ g given at a single time point.

PVA hydrogel systems were prepared with selected parameters in an effort to tailor the release of Serp-1 to the above stated conditions. The samples were fabricated with two freeze-thaw cycles, 10 wt% PVA solution, 0.1  $^{\circ}$ C/min freezing and thawing rates, and loaded with about 200  $\mu$ g Serp-1. FIGURE 4.29 demonstrates the results of this set of conditions. Over approximately 100 hours, the goal of 125  $\mu$ g of Serp-1 was achieved. This is only one example of a possible therapeutic scenario, there are other possibilities although the ideal scenario in a clinical setting is still unknown. The results suggest Serp-1 delivery from PVA hydrogel can be extended for several days by adjusting the processing parameters and protein loading. To increase the duration of delivery, parameters such as freezing and thawing rates and number of cycles can be tuned to control the physical crosslinking of the hydrogel which ultimately controls the diffusion of Serp-1 out of the matrix. Therefore, once a therapeutic dosage and timeframe are identified for a clinical setting, the level of Serp-1 delivery can easily be adjusted.



**FIGURE 4.29.** *In vitro* release profile of Serp-1 from PVA at a therapeutic loading into a buffered medium. The PVA samples were prepared using two freeze-thaw cycles, 10 wt% PVA solution, 0.1 °C/min freezing and thawing rates, with about 200  $\mu\text{g}$  Serp-1 loaded. Data represents the average of 3 samples and the error bars are the standard deviation.

#### 4.3.8 Serp-1 Release into Human Whole Blood

Up to now, the Serp-1 release experiments have been performed *in vitro* using buffers as the release medium. In an effort to create a more realistic biological environment to test the controlled release properties of Serp-1 from PVA hydrogels, human whole blood was investigated as a release medium.

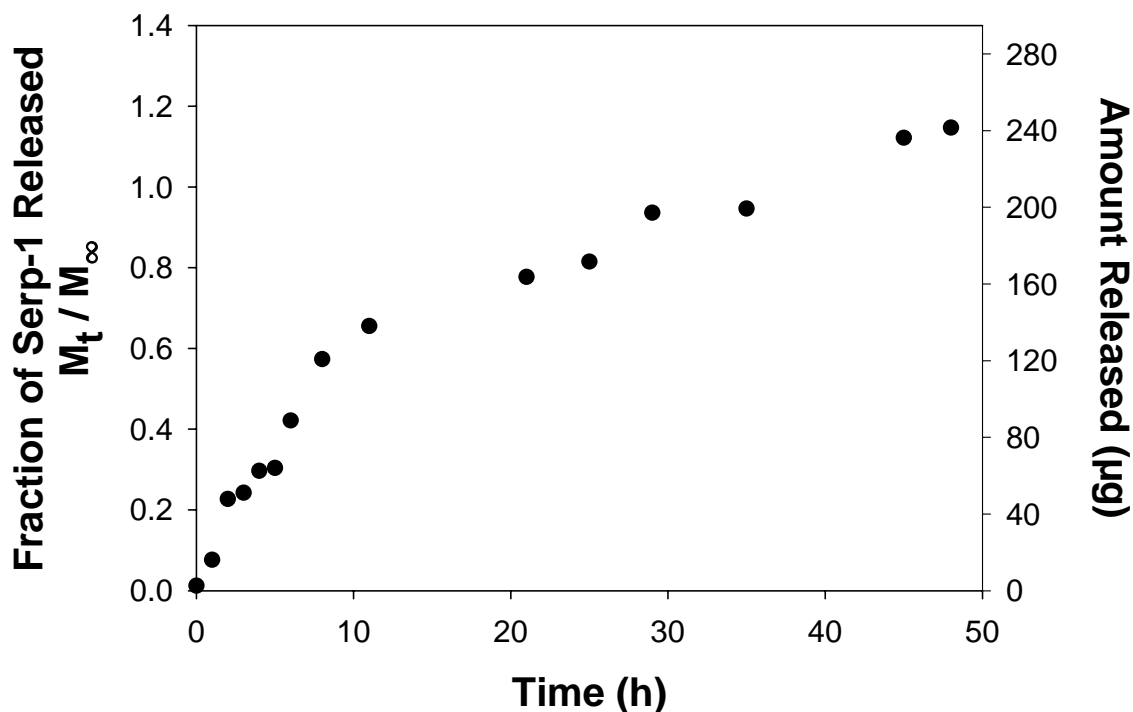
Using the same Serp-1 loading and processing conditions as the previous study at a therapeutic dosage, an *in vitro* release experiment was conducted in human whole blood instead of buffer. FIGURE 4.30 displays the release profile for this case where the data is



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preliminary and an average of 2 samples is shown. The most obvious difference from this study compared to the previous study in buffer, is that more Serp-1 is released in a shorter period of time. About 60 % of the loaded Serp-1 was released when buffer was used a release medium. However, in human whole blood 100 % release was observed. Also, the release reached completion in about 50 hours, half the time monitored in FIGURE 4.29.

The results, although preliminary, of this study suggest there may be a difference in release behaviour for the two different mediums. This becomes an important factor for the control of release, since the human whole blood represents a much more realistic biological setting. Possible reasons for the difference in release rate and amount may include a difference in ionic strength between the buffer and whole blood. As demonstrated in a previous section of this thesis using BSA as a model protein, the ionic strength of the release medium plays a role in the amount of protein released. If the ionic strength of the buffer is higher than the whole blood, then this may account for part of the decreased release in the buffer. Also, it may be that Serp-1 has more activity in whole blood and therefore was more easily detected, causing an increase in readings. Further studies need to be conducted to determine if this truly is a concern and that there is such a large difference in outcomes between the two release mediums.



**FIGURE 4.30.** *In vitro* release profile of Serp-1 from PVA at a therapeutic loading into human whole blood. The PVA samples were prepared using two freeze-thaw cycles, 10 wt% PVA solution, 0.1 °C/min freezing and thawing rates, with about 200  $\mu\text{g}$  Serp-1 loaded. Data is preliminary and is the average of 2 samples.

#### 4.4 DESIGN OF A CONTROLLED RELEASE SYSTEM

The previous sections of this chapter have discussed many results which are relevant when considering the design of a controlled release system. In particular, a controlled release system of Serp-1 protein from PVA hydrogels was of interest. Fundamental studies using a model protein, BSA, were done to investigate key behaviours of protein release from PVA hydrogel. This was followed by a systematic study of the properties of Serp-1 release from PVA hydrogel as a function of the processing parameters, including the number of freeze-thaw cycles, PVA concentration, freezing and thawing rates, as well as protein loading. Combining all this information

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together provides insight for designing a controlled release system for the reduction of restenosis.

For local restenosis reduction the idea is to implant the material locally to the site of vascular injury, whether as a coating for a DES or a cuff or patch implanted in the artery. Consequently, the drug delivery system will be in contact with the blood stream. *In vivo*, blood then becomes the equivalent to the release medium used in the *in vitro* studies. Blood contains many ions which could potentially contribute to an osmotic pressure effect on the hydrogel system which influences the release properties as demonstrated in section 4.1.3. The physiological ionic strength of blood is 0.15 M, thus as a release medium it can be most closely compared to the results with 1X PBS which has an ionic strength of 0.17 M. The results have demonstrated that with 1X PBS as a release medium there is a decrease in protein released amount due to shrinking of the PVA structure as water is drawn out of the hydrogel and an effective increase in PVA concentration. Therefore, it should be anticipated when a PVA hydrogel controlled release system is placed in a physiological environment this effect will be observed. For Serp-1, the *in vitro* studies were conducted with 1X PBS which has an ionic strength close to physiological conditions. Hence, the results reflect protein release amounts that could be expected *in vivo*.

The Serp-1 studies also demonstrated that a portion of the protein remains trapped within the PVA matrix. This is partially caused by osmotic pressure from the ionic strength of the release medium compared to the solvent of the hydrogel, leading to a decrease in release amount as discussed above. It is thought that there are other contributing factors to the high percentage of Serp-1 that remains trapped in the matrix, such as the interaction between Serp-1 and PVA. Regardless, it is important to account for a portion of Serp-1 remaining in the controlled release platform when developing a therapeutic system. The portion of Serp-1 remaining in PVA will depend on the processing conditions and the resulting hydrogel, thus, the percentage remaining needs to be determined for the particular set of parameters required for a clinically therapeutic system.

When designing a controlled release system for Serp-1, the current methods of treatment must be considered and improved on. Clinical trials of Serp-1 involve giving an infusion of Serp-1 immediately following angioplasty and stent deployment [66]. By infusing Serp-1 locally, the protein is delivered directly to the site of injury. However, the local concentration of Serp-1 will spike and then rapidly diminish as it is washed out and the plasma half-life is only about an hour. This is where a controlled release system can improve upon the current delivery method of infusion. By gradually releasing Serp-1 over time the local concentration can be maintained at a constant level and for a longer period of time. Since Serp-1 is still being investigated in clinical trials, the exact treatment protocols have yet to be established. Having options for a controlled release system will be of benefit, so that once the desired timeframe of delivery and dosage of Serp-1 are known, the system can be tailored to meet the requirements.

In this thesis it has been demonstrated that from a matrix-type system there are several processing parameters that can be varied to tune the PVA/Serp-1 system. The number of freeze-thaw cycles, PVA concentration, freezing and thawing rates, and protein loading can all be adjusted to tailor the system by release rate and release amount. Additionally, to adjust the release kinetics, a section in this thesis also discussed the possibility of using a two-layer reservoir-type system to release protein from PVA. Although for these studies BSA was used as a model protein, the results can be applied to Serp-1 and other water-soluble proteins. Depending on the intended application, two types of release kinetics, Fick's first and second laws, have been demonstrated. If a steady release rate is desired to maintain a constant concentration of protein delivered, the reservoir-type system should be used. If the application is more flexible and the release rate does not have to be constant, but a gradual method of delivery is still pertinent, the matrix-type system should be selected. For Serp-1, the matrix-type PVA system may be sufficient. It is thought that earlier time points of delivery are most important as injections of Serp-1 closest to the time of injury have proven to be the most effective at reducing restenosis [76]. With the PVA/Serp-1 release system, that follows diffusion controlled release, the protein release rate is faster at earlier times and slows down as

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time progresses. This makes it a good candidate for an application that requires the highest concentration of drug delivery at early times.

For the application of a DES, the PVA/Serp-1 system would be a relevant coating material. It likely does not have the mechanical properties to be a fully polymeric stent, but could coat a metallic stent. This would allow for controlled delivery of Serp-1 to the local site of vascular injury when it is required the most as a stent is deployed.

PVA hydrogel by the freeze-thaw method is an excellent controlled release matrix for protein including Serp-1. Water-soluble protein delivery is most efficient in a hydrophilic environment as they will not readily diffuse through a hydrophobic matrix. Other hydrogels are also potential delivery matrices for protein, however, they must have a means to adjust the crosslinking density or other parameters which influence the rate of drug release without denaturing the protein by the use of chemical agents. This is where the PVA system proves to be advantageous with the physical crosslinking methods and many processing parameters which allow the system tunable. PVA hydrogel would be an applicable delivery matrix for other water-soluble proteins. As studied with BSA and Serp-1, the trends in release properties would be similar, although there may be some differences with Serp-1 and BSA. It would be expected for other proteins the release would be diffusion controlled and the system easily tuned in the same manner as with Serp-1 or BSA.

## Chapter 5

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# CONCLUSIONS AND FUTURE WORK

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### 5.1 CONCLUSIONS

The focus of this thesis was the design of a PVA hydrogel controlled release system to deliver the Serp-1 protein for the management of restenosis. Serp-1 has shown promise in animal studies as being an effective anti-inflammatory agent and is currently under investigation in clinical trials. Serp-1 has a relatively short half-life *in vivo* and may require multiple injections or infusions for successful use, thus a more efficient means of delivery to prolong the activity of it is desired. PVA hydrogels prepared by the freeze-thaw method were investigated as a possible delivery matrix.

BSA was used a model protein to investigate the diffusion properties of PVA hydrogel. The controlled release properties were examined as a function of the number of freeze-thaw cycles and a decreasing trend in diffusion coefficient was observed with increasing number of cycles.

BSA was also used as a model protein to demonstrate the release profile of a matrix-type system versus a reservoir-type system. The reservoir-type system was found to follow Fick's first law with zero-order kinetics, while the traditional matrix-type system follows Fick's second law of diffusion. Therefore, the reservoir-type system provides an additional option for delivery kinetics. It was also established that the rate of protein release from the reservoir-type system was dependent on the thickness of the

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outer barrier of protein-free PVA. The thicker the barrier layer, the slower the rate of release of BSA was.

BSA was further used as a model to study the effect of the release medium's ionic strength on the amount of protein released. For clinical application of biomaterials this effect is important since body fluid contains a high ion concentration. It was observed that using a release medium such as PBS instead of water, induces an osmotic pressure in the hydrogel comprised mainly of water. The effect of this draws water from the hydrogel, causing the hydrogel structure to shrink and causes an increase in apparent PVA concentration. Effectively this reduces the mesh space available for diffusion and a reduction in protein release was observed with increasing release medium ionic strength.

The release of Serp-1 from PVA hydrogels was characterized. It was found that Serp-1 also follows diffusion controlled release and a range of control can be attributed to the processing parameters selected. Analysis of the release process was complicated by a large percentage, up to 50 %, of Serp-1 remaining trapped in the PVA matrix, due to a combination of factors including a stronger interaction with PVA and reduced mesh space for diffusion from osmotic pressure induced by the release medium used. Diffusion coefficients must be corrected to account for the percentage of loaded Serp-1 that remains trapped in the PVA matrix. By correcting the diffusion coefficients it was demonstrated the release rate of Serp-1 is comparable to that of BSA. Increasing the number of freeze-thaw cycles again demonstrated a decrease in diffusion coefficient. Increasing the PVA solution concentration also caused a decrease in Serp-1 release. When the freezing or thawing rate was increased, the release rate of Serp-1 increased. Increasing the protein loading of the hydrogel system also increased the amount of Serp-1 release.

Using PVA hydrogels by the freeze-thaw method appears to be a viable approach for Serp-1 delivery. The selection of processing parameters provides a means of control over the release properties. A therapeutic level of delivery can be achieved by selecting the appropriate processing parameters and increasing the drug loading accordingly. The

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release kinetics can be manipulated by selecting a one-layer matrix-type system or two-layer reservoir-type system depending on whether a constant release rate is required.

## 5.2 FUTURE WORK

The work of this thesis has demonstrated the *in vitro* controlled release properties of the PVA/Serp-1 system. However, the effectiveness of this system *in vivo* is not yet known. Ideally, to validate the use of the PVA/Serp-1 system, small animal studies must be done to determine its effect on the restenotic process. By implanting the material in an animal model, the release can be monitored to estimate the level of Serp-1 present at the local site. If Serp-1 remains detectable for a period greater than that from an infusion of Serp-1, then the system should be considered a success. The anti-inflammatory activity of the system should also be considered by monitoring the presence of immune cells surrounding the local implant site. Ideally, the slow controlled delivery of Serp-1 will reduce the local immune response. From the results of the initial animal studies, the controlled release properties can be tailored accordingly using the parameters studied in this thesis to adjust the release to be most effective.

From previous animal studies using injections, Serp-1 has been shown to be most effective for preventing restenosis at time points closest to the onset of injury. Therefore, diffusion controlled release may be most appropriate with the majority of release predominating in the early hours of release. However, should the clinical application turn out to be more suited to zero-order kinetics or a constant maintenance dosage is beneficial, the multi-layer system demonstrated in this thesis using BSA as a model protein, could be implemented.

Serp-1 delivery could potentially be accompanied by the delivery of another complementary therapeutic agent. Especially for the application of DESs, there are many potential biological targets. While Serp-1 may effectively manage restenosis in the shorter term of a day or two, another therapeutic agent may be required to maintain the stent's patency. For example, current treatment includes long term anti-platelet therapy



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following stent implantation. It may be possible to incorporate a second anti-restenotic agent or anti-platelet agent in the PVA system along with Serp-1. For this, a multi-layer structure might be best to separate each molecule into an individual layer of PVA with separate release kinetics. Potentially an anti-platelet drug could be embedded in the inner layer to be released by zero-order kinetics over a longer and gradual timeframe, while Serp-1 could be delivered from the outer layer in a shorter diffusion controlled release profile. The compatibility of two agents releasing from a multi-layered PVA system has yet to be investigated, but could provide a great opportunity for a multi-targeted approach to reducing restenosis.

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**Appendix A**

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**ELISA PROTOCOL**

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## SERP-1 SANDWICH ELISA PROCEDURE

### MATERIALS

PBS (10X, 1 L)	2.4 g Potassium Phosphate (KH <sub>2</sub> PO <sub>4</sub> ) 2.0 g Potassium Chloride (KCl) 80.0 g Sodium Chloride (NaCl) 26.8 g Sodium Phosphate, Dibasic Heptahydrate (Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O) Add chemicals to ddH <sub>2</sub> O for a final volume of 1 L. Expiry is 1 year from date prepared.
Carbonate Coating	Carbonate-bicarbonate buffer capsules (Sigma, C-3041) Buffer (CCB) Dissolve one capsule in 100 mL deionized water to yield a 0.05 M buffer, pH9.6 at 25 °C. Store at 4 °C and expiry is 1 year from date prepared.
Blocking Buffer (1 L)	100 mL of 10X PBS 20 g BSA 0.5 mL Tween-20 Add chemicals to ddH <sub>2</sub> O for a final volume of 1 L. Aliquot and store at -20 °C up to 1 year from date prepared.
Wash Buffer (1 L)	0.05 % Tween-20 in PBS  100 mL of 10X PBS 0.5 mL Tween-20 Add chemicals to ddH <sub>2</sub> O for a final volume of 1 L. Store at room temperature.
Assay Buffer (1 L)	1 % BSA/0.025 % Tween-20 in PBS (50 % blocking buffer + 50 % wash buffer) Aliquot and store at -20 °C. Expiry is 1 year from date prepared.
Stop Solution	2 N H <sub>2</sub> SO <sub>4</sub> Add 27.75 mL 18 N H <sub>2</sub> SO <sub>4</sub> in to 222.25 mL ddH <sub>2</sub> O. Store at room temperature in acid cabinet.
Capture Antibody	ATD11, Serp-1 mAb (Viron Therapeutics Inc.)
Detection Antibody	bAXB7.9, Serp-1 mAb (Viron Therapeutics Inc.)

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HRP Streptavidin	P21124 (Pierce Biotechnology, Inc. (Thermo Fisher Scientific))
TMB Substrate Kit	DY999 (R&D Systems)

## **METHODS**

**Step 1:** Coat - Capture mAb (ATD11), 1.6 µg/mL on 96 well plate (Corning 3590)

- For one plate, 7.33 µL of 1.2 mg/mL ATD11 in 5.5 mL CCB, dispense 50 µL per well
- Cover plate with parafilm and incubate in fridge (4 °C) overnight or up to one week

Remove coat antibody by inverting plate over sink and blotting plate on paper towel

**Step 2:** Block - Blocking buffer

- Dispense 200 µL per well
- Incubate at room temperature for one hour

Wash 3X – Wash buffer, 200 µL per well

**Step 3:** Standards and samples - Dilute standards and samples in assay buffer

- For standard curve, dilute purified Serp-1 by adding 450 µL of assay buffer to a 50 µL aliquot of 2.0 µg/mL Serp-1 to get 200 ng/mL Serp-1
- In first two columns of the polypropylene dilution plate, dilute Serp-1 standard from 0-24 ng/mL as follows

Final Concentration (ng/mL)	Assay Buffer (µL)	200 ng/mL Serp-1 (µL)
24	176	24
20	180	20
16	184	16
12	188	12
8	192	8
4	196	4
2	198	2
0	200	0

- For samples, dilute serially using assay buffer in remaining columns of dilution plate using wet tip method
- All dilutions are done in polypropylene dilution plate and then transferred to polystyrene assay plate, 50 µL per well, running all samples in duplicate
- Incubate at room temperature for one hour

Wash 3X – Wash buffer, 200 µL per well

**Step 4:** Biotinylation - Detection mAb (bAXB7.9), 0.1 µg/mL

- For one plate, 55 µL of 10 µg/mL bAXB7.9 in 5.5 mL assay buffer, dispense 50 µL/well
-

- Incubate at room temperature for one hour

Wash 3X – Wash buffer, 200  $\mu$ L per well

**Step 5:** Biotin detection - HRP Streptavidin, 0.25  $\mu$ g/mL

- Add 500  $\mu$ l of 5  $\mu$ g/ml HRP Streptavidin to 10 mL assay buffer (enough for 2 plates), dispense 50  $\mu$ L per well
- Incubate at room temperature for one hour

Wash 3X – Wash buffer, 200  $\mu$ L per well

**Step 6:** Colour development - TMB kit

- Mix 5.5 mL vial A (stabilized hydrogen peroxide) and 5.5 mL vial B (stabilized tetramethylbenzidine), use immediately by dispensing 100  $\mu$ L per well
- Incubate at room temperature for only 4 minutes

**Step 7:** Stop - 2 N H<sub>2</sub>SO<sub>4</sub>

- 100  $\mu$ L per well pipetted in the same order as previous step
- Read plate immediately

**Step 8:** Read - Microplate reader at 450 nm

- Zero plate reader by reading blank wells and set the average value to first well
  - Turn on the autoreader and manually maneuver plate to read each well in order of pipetting
  - Output data to text file and import into spreadsheet
-

**Appendix B**

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**SDS-PAGE PROTOCOL**

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## SDS-PAGE PROCEDURE

### MATERIALS

SDS-PAGE Sample Buffer (2X, 100 mL)	20 mL of Glycerol 4 g Sodium Dodecyl Sulfate (SDS) 200 mg Bromophenol Blue 20 mL 1 M Dithiothreitol 10 mL 1 M Tris Add chemicals to ddH <sub>2</sub> O for a final volume of 100 mL.
SDS-PAGE Running Buffer (10X, 1 L)	30 g of Tris 144 g of Glycine 10 g SDS Add chemicals to ddH <sub>2</sub> O for a final volume of 1 L.
Serp-1 Control	1.8 mg/mL Serp-1 (Viron Therapeutics, Inc.)

### METHODS

#### **Step 1:** Prepare samples and controls

- Dilute samples in SDS-PAGE sample buffer
- Serp-1 (1.8 mg/mL) control dilute in SDS-PAGE sample buffer
- Centrifuge samples briefly to mix and heat at 95 °C for 5 min

#### **Step 2:** Load gel

- Place pre-made gels (Bio-Rad 161-1105) in electrophoresis box and fill with 1X SDS-PAGE running buffer
- Load 10 µL standard (Bio-Rad 161-0376) as marker in first lane
- Pipette samples and controls at volumes to achieve appropriate loading in remaining lanes

#### **Step 3:** Electrophoresis

- Run gel at constant voltage (200 V) using Bio-Rad Power Pack 300 for 25 min
  - Reduce voltage to 120 V and run another 25 min
  - Remove gel from assembly and rinse with dH<sub>2</sub>O
-



## **Appendix C**

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# **WESTERN BLOT PROTOCOL**

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## WESTERN BLOT PROCEDURE FOR SERP-1

### MATERIALS

Transfer Buffer (10X, 1 L) (without methanol)	30 g of Tris 144 g of Glycine Add chemicals to ddH <sub>2</sub> O for a final volume of 1 L.
Transfer Buffer (1X, 1 L) (with methanol)	100 mL of 10X transfer buffer 200 mL Methanol Add chemicals to ddH <sub>2</sub> O for a final volume of 1 L.
PBS (10X, 1 L)	2.4 g Potassium Phosphate (KH <sub>2</sub> PO <sub>4</sub> ) 2.0 g Potassium Chloride (KCl) 80.0 g Sodium Chloride (NaCl) 26.8 g Sodium Phosphate, Dibasic Heptahydrate (Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O) Add chemicals to ddH <sub>2</sub> O for a final volume of 1 L.
Blocking Buffer (100 mL)	5 g of Instant Skim Milk Powder 0.2 mL of Tween-20 10 mL of 10X PBS 89.8 mL ddH <sub>2</sub> O
Wash Buffer (1 L)	0.2 % Tween-20 in PBS 100 mL of 10X PBS 2 mL Tween-20 Add chemicals to ddH <sub>2</sub> O for a final volume of 1 L.
Primary Antibody	AQH9.6, Serp-1 mAb in 50 % glycerol (Viron Therapeutics Inc.)
Secondary Antibody	Goat Anti-mouse Antibody (Bio-Rad Laboratories, Inc)
Western Blotting Detection Reagent	1059243 (Amersham Life Science)

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## **METHODS**

### **Step 1:** Transfer (blot)

- Pour chilled 1X transfer buffer into both sides of blot assembly tray
- Presoak filter paper and nitrocellulose membrane (Amersham RPN303E) in 1X transfer buffer
- Assemble sandwich with three pieces of filter paper, then electrophoresis gel, then membrane, then three more pieces of filter paper and roll out bubbles
- Close cassette and pour off excess buffer
- Run blot at constant current (480 mA) for 40 min using Bio-Rad Power Pack 200

### **Step 2:** Blocking buffer

- Place membrane in blocking buffer
- Incubate at room temperature on rocker for one hour

### **Step 3:** Primary antibody – mAb AQH9.6 (50 % glycerol)

- Dilute first antibody (7  $\mu$ L) in blocking buffer (20 mL)
- Incubate at room temperature on rocker for one hour

Wash 3X – Place membrane in wash buffer for 5-10 min on rocker

### **Step 4:** Secondary antibody – HRP goat anti-mouse

- Dilute second antibody (7  $\mu$ L) in blocking buffer (20 mL)
- Add in 4  $\mu$ L antibody for Bio-Rad marker ladder (Bio-Rad 310003685, Precision StrepTactin-HRP Conjugate)
- Incubate at 4 °C overnight on rocker

Wash 3X – Place membrane in wash buffer for 5-10 min on rocker

### **Step 5:** Detection solution

- Coat membrane with detection solution (1 mL A + 1 mL B) for 1-2 min
- Blot dry on Kim wipe
- Place membrane between two plastic sheets in film cassette

### **Step 6:** Film development

- In dark room, add film to cassette with membrane for 5 s exposure
- Run film through developer

### **Step 7:** Bio-Rad Gel Doc system

- Add film to Gel Doc system and turn on lights
  - Open software, adjust image and save as a TIFF file
-

**Appendix D**

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*Controlled Delivery of Serp-1 Protein from Poly(vinyl alcohol) Hydrogel*  
Supervisor: Dr. Wankei Wan
- 2003      **Bachelor of Engineering (BEng)**  
Dalhousie University, Halifax, Nova Scotia  
Biosystems Engineering: Biomachines and Robotics Option
- 2000      **Bachelor of Science (BSc)**  
Saint Mary's University, Halifax, Nova Scotia  
Major: Biology, Minor: Chemistry

### AWARDS

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- 2009              Canadian Biomaterials Society Conference Travel Award (\$500)
- 2005 – 2008      Western Graduate Research Scholarship (WGRS) (\$6600 per year)
- 2005 - 2007      CIHR Training Award in Vascular Research (\$10,000 per year)

### RELATED WORK EXPERIENCE

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- 2006 – 2008      **Graduate Teaching Assistant in Biomaterials Engineering**  
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- 2006 – 2007      **Graduate Teaching Assistant in Introduction to Biomedical Engineering**  
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- 2003 – 2005      **Graduate Teaching Assistant in Industrial Organic Chemistry**  
Department of Chemical and Biochemical Engineering  
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Supervisor: Dr. Paul Charpentier

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## CONFERENCE PRESENTATIONS

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- Kennedy, K.L.**, Wan, W.K. (2010) Controlled Release of Protein from Poly(vinyl alcohol) Hydrogels. Poster Presentation at the 28th Annual Meeting of the Canadian Biomaterials Society, Kingston, Ontario.
- Kennedy, K.L.**, Lucas, A.R. and Wan, W.K. (2009) Controlled Release of an Anti-inflammatory Protein. Oral Presentation at the 27th Annual Meeting of the Canadian Biomaterials Society, Quebec, Quebec.
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-