In vitro and in vivo studies of controlled release of novel peptide from in situ polymer precipitation delivery system

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IN VITRO AND IN VIVO STUDIES OF
CONTROLLED RELEASE OF NOVEL PEPTIDE
SITE POLYMER PRECIPITATION DELIVERY S

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Abstract

The *in situ* polymer precipitation delivery system has generated much interest in the last two decades. The ease of manufacturing and delivery of this system are its key advantages that have contributed to its increasing preference for drug delivery. Although much research has been performed on this system, the studies that have translated into animal and human data are limited, and the release rates are much lower than desired.

This study aims to investigate and understand the release mechanism involved in peptide release from polymer precipitation delivery system. This study aims to establish a relationship between *in vitro* derived data. The ultimate aim is to identify a delivery system that can be used to deliver therapeutic peptides for the treatment of heart failure conditions.

This study can be divided into three parts.

First, *in vitro* studies were performed to characterise systemically the release of peptide and its efficacy at achieving the prolonged release. Various formulation parameters were tested, including polymer concentration, polymer type, drug loading and injected volume. The system using the gel formulation PLGA / 40% NMP / 20% triacetin was the most suitable for achieving the desired peptide release profile. Investigation of the effect of polymer concentration on the release profile and shell structure formation morphology were also performed.

solvent efflux and shell structure formation developed from and to be affected the drug release profile.

Second, ~~in vivo~~ studies were performed on both healthy and diseased rats. As a preliminary feasibility study, the peptide polymerase precipitation delivery system was performed on healthy rats. The results suggested the system was efficacious. In addition, the peptide bioactivity was preserved throughout the prescribed study. The results from the diseased rat model supported the feasibility studies. The acute myocardial infarction rat model was established by ventricular ligation. As expected, the preservation of peptide bioactivity was maintained at the study endpoint. More important, in this disease model, the peptide effectively attenuated the heart failure syndrome, demonstrating the preservation of cardiac functionality and structural integrity.

Last, a simplified *in vivo* correlation was established. Furthermore, it was established that the shape of the injected depot contributed to the release profile. As such, proper correlation between *in vivo* and *in vitro* data during the solidification phase of the injected gel could not be established. Until complete solidification of the polymer depot, a steady peptide release was observed in plasma. The estimated peptide plasma concentration was consistently 2.5x higher than that of the actual measured value, which was attributed to the simplified single compartment diffusion model. To establish this relationship.

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Chapter Introduction

1.1 Project Motivation

Worldwide, cardiovascular diseases remain the leading cause of death. Cardiovascular diseases come in various forms and usually include a syndrome of heart failure. Heart Failure (HF) is a condition in which the heart cannot supply sufficient blood flow to the needs of the body. Characteristics of HF include functional and structural changes in the heart, endothelial dysfunction with vasoconstriction, sodium and water retention, and neurohumoral activation.

In United States, approximately 2% of the population suffer from HF, often known as congestive HF with congestive HF accounting for about 1 million of the annual hospital admissions. These admissions are based on HF as a primary diagnosis. Another 2 million are admitted with heart failure as a secondary diagnosis. While it is well known that reperfusion therapy received when in hospital can improve outcomes, a third of these patients get readmitted within 90 days after the initial admission.

The increasing occurrence of HF is largely due to lifestyle factors such as over indulgence of oily foods, sedentary work life, lack of exercise, and aging. These are all contributing factors to the development of HF. HF is a chronic condition that requires long-term management.

always escalate on patients with existing conditions such as hypertension, coronary artery disease, diabetes mellitus, family history of obesity.

Indeed, with the increasing prevalence of lifestyle change, the of HF is most likely to further increase. Consequently, this will certainly add on additional stress on overstretched public health resources.

Depending on the stages of HF, there are different treatment options available to the patients. Generally, in the early stages, treatments are more preventive. Treatments such as long term oral medications and lifestyle changes are usually recommended to patients in the early stages of HF where medications may be able to effectively control the syndrome. In the later stages of HF, surgical interventions may be called upon. However, in the later stages of HF, medications may not be able to completely cure HF. In this case, and preventing the worsening of heart failure is still the best option.

There are various medications that can be utilized for the treatment of heart failure. One of the ways is to administer exogenous natriuretic peptides. Natriuretic Peptides (NPs) are known to have beneficial effects on heart failure. Several synthetic versions of NP have been developed, such as Carperitide (synthetic NP) and Nesiritide (synthetic NP). Both have been approved for the treatment of acutely decompensated HF.

respectively. The use of synthetic NPs, which encompasses the variants, has its limitations [4-7].

1.2 Problem Statement

Presently, administration of these peptides during patient treatment to control the condition discharge, patients are required to perform frequent injections of these peptides as part of the long term the heart condition. However, these peptides typically have a half-life of 420min [8, 9]. As such, multiple peptide injection is needed. This greatly reduces the patients quality of life and is a poor compliance. Statistics show that despite the improvements in HF compliance inpatient hospitalization treatment, there is still a high occurrence and mortality rate associated with discharged patients. It has been suggested that one possible reason for high readmission and mortality rate is poor compliance [10].

In order to improve on patient compliance, novel therapeutic strategies such as a peptide delivery system which could provide sustained release over a longer duration. This delivery system should maintain the structural and conformational stability of the peptide, protecting it from degradation while maintaining a sustained release over an extended period. If achieved, this delivery system would have benefits such as improved compliance and quality of life while reducing overall healthcare costs.

1.3 Objectives and Scope

Researchers at Mayo Clinic have engineered a novel chimeric natriuretic peptide, Cenderitide, which is currently undergoing clinical trials. This peptide combines the full native aminoterminal structure of C-type natriuretic peptide (CNP) with the C-terminus of angiotensin-converting enzyme (ACE) inhibitor, lisinopril. The resultant peptide, Cenderitide, possesses properties of both peptides. It is shown that Cenderitide, which acts on both Natriuretic Peptide Receptor (NPR) and Angiotensin Receptor (ARB), can avoid the hypotensive and renal effects of the natriuretic moiety while maintaining the cardiorenal protective properties [1417].

This thesis seeks to investigate on the possibility of formulating precipitation delivery systems for the delivery of Cenderitide peptide. The hypothesis is that a better control over the initial burst (low initial release) and subsequent peptide release profile can be achieved by manipulation of injection formulation characteristics such as solvent content, drug loading and volume.

To verify the stated hypothesis, the work has been divided into the following objectives:

First objective: To study and understand the effect of various solutions on the controlled release of Cenderitide peptide in *in vitro* studies.

Second objective: To identify one or more formulations that can achieve the desired release profile using *in vitro* studies.

Third objective: To validate the results on the selected formulation(s) in *in vivo* model. This objective can be divided into two parts: a feasibility study in normal healthy rats and efficacy of delivery in a disease model.

model. The former serves as a proof of concept for the delivery of the peptide. The latter aims at elucidating preservation of cardiac functions.

Fourth objective: To try to establish a correlation for the gelling system.

1.4 Thesis Novelty

Although the idea of forming implants has been around for decades, studies have reported on controlled release of peptides to further the understanding of peptide release mechanism from such particular forming polymer precipitation delivery system. Certain new peptide which is currently still undergoing clinical studies, so the work lies in the identification of key factors that minimize peptide incorporation in the gelling system. In this work we also show time *in vivo* effects of a sustained delivery system for Cenderitide, a natriuretic peptide used to control the effects of ventricular heart attack.

Chapter 2 Literature Review

2.1 Heart Failure Disease State

Heart failure (HF) syndrome is associated with impaired cardiac functions. The most frequent representation of HF is the heart's inability to pump sufficient blood volume to meet the body needs. Typically, HF is characterised by functional and structural changes, including ventricular remodeling, vascular dysfunction with vasoconstriction, sodium and water retention, kidney, and neurohumoral activation.

The heart is very susceptible to damage from a variety of causes. In these circumstances, heart health begins to deteriorate gradually, leading to heart failure syndrome. As the condition of the heart deteriorates, the neurohormonal pathways that release specific hormones are activated. These hormones, such as renin and angiotensin, are released into the bloodstream to increase the circulating blood volume and to reduce the heart's workload by relaxing the vascular wall. Whereas these hormones initially have beneficial effects on unloading the heart, prolonged activation leads to adverse effects, worsening the HF syndrome. For example, the prolonged circulation of angiotensin and vasopressin leads to a decrease in renal perfusion. Consequently, water accumulates gradually in the body. Elevated aldosterone levels also contribute to fluid retention.

to sodium and water, and more crucially, this hormone increases fibrosis, resulting in the worsening of cardiac remodelling.

If these conditions are unchecked, left ventricle (LV) remodelling, myocyte hypertrophy and LV elongation occurs, enlarging volume. This meant that although the stroke volume is increased, there is an increase in the ejection fraction (This is due to the lower efficacy of the remodeled LV wall).

Fortunately, not all of the hormones released have an adverse effect on the heart. Another key osmoregulatory hormone, known as natriuretic peptide, is released to regulate sodium, blood volume and blood pressure. These natriuretic peptides also exhibit cardiac and renal effects. This will be discussed in more detail in the next section.

Many different causes and disease states that damage or overwork the heart can result in HF. HF can be categorised into systolic and diastolic HF. In systolic HF, the heart is incapable of performing a proper contraction, and thus, it cannot pump efficiently enough to pump sufficient blood to all parts of the body. This leads to the insufficient delivery of nutrients needed for the heart's metabolism. In diastolic HF, the heart is unable to fill with blood properly during cardiac relaxation or experiences abnormal ventricular filling. This results in less blood sent to the lungs for oxygenation, resulting in an insufficient amount of oxygenated blood being diverted back to the left ventricle.

of HF patients suffer from the former condition, there are a few cases where both conditions are present concurrently.

Figure 2-1: Illustration of the Types of Heart Failure
(Diagram taken from The MERCK Manual, Home Health Handbook, 2018)

2.1.1. Epidemiology

In the USA and most developed countries, HF is the most common cardiovascular disease. In the United States alone, approximately 5.8 million people suffer from some form of HF. Although children and adults can suffer from HF, the prevalence of HF increases with age. With approximately 550,000 new cases identified each year and more than a million hospitalisations annually, HF is the leading cause of hospitalisation in elderly people.

2.1.2. Etiology

Many factors, such as congenital heart defects, accidental heart trauma, and coronary artery disease, have been identified as causes of heart failure. The most common cause of HF is coronary artery disease.

failure are coronary heart disease, high blood pressure, and diabetes, which are direct results of lifestyles.

Coronary heart disease is a condition in which fatty substances are deposited on the inner lumen of the coronary arteries. These deposits, as plaque, narrow the arterial lumen. This condition, known as atherosclerosis, affects the flow of oxygenated blood. This diminished blood supply to the myocardial cells results in heart failure.

Figure 2: Illustration of Atherosclerosis
(Diagram taken from National Heart Lung and Blood Institute, 2000)

Blood pressure is the measurement of the force acting against the walls of the arteries. In patients with persistent high blood pressure, this condition weakens the arteries and can lead to plaque build-up.

Diabetes is a condition in which consistently high blood glucose levels circulate in the body. High blood glucose levels can be caused by the body not producing enough insulin or utilising insulin properly. Persistent high blood glucose levels can lead to complications.

damage and weaken the heart muscle and blood vessels around the heart, leading to HF syndrome.

Heart injuries leading to HF can also result from my radiatic treatments for cancer, thyroid disorder, alcohol or drug abuse, and vitamin E. Common congenital heart defects that contribute to HF are cardiomyopathy, heart valve disease and arrhythmias.

The New York Heart Association (NYHA) has published a table to classify the stage of heart failure in accordance with heart function and the HF stages based on everyday activities and the patient's symptoms.

Table 2-1: Classification of Chronic Heart Failure
New York Heart Association, Heart Failure Society of America

Depending on the patient's heart failure classification, different treatment strategies are employed. For Class I patients, lifestyle changes and medications are used to prevent the development of HF. For Class II patients, the indication for treatment is more specific.

disease require a strategy to prevent LV remodelling. In addition, medications are introduced in patients with congestive heart failure to slow the deterioration of HF syndrome. For patients diagnosed with CHF, typically employed are, high technology therapies or mechanical supports in addition to medications.

2.1.1. Acute Myocardial Infarction

Acute myocardial infarction (AMI) is a common clinical outcome for various cardiovascular diseases. It begins when myocardial ischemia occurs due to coronary artery occlusion. The limited or lack of oxygenated blood to the heart over an extended period leads to irreversible damage to myocardial cells. Typically, following myocardial infarction, the heart starts to deteriorate, gradually leading to heart failure.

Figure 2.3: Illustration of Formation of Acute Myocardial Infarction
(Diagram taken from National Heart Lung and Blood Institute, 2010)

Of the main diseases causing HF syndrome, myocardial infarction is the leading cause of death worldwide. In the United States, an estimated 450,000 people die from coronary disease every year. The extent of the infarction is determined by the following three key factors:

- 1) Occlusion location in the coronary artery. Generally, when the occlusion is located higher in the main branch of the coronary artery, the infarcted area is larger.
- 2) Length of time of the occlusion. The longer the blockage in the coronary artery, the longer it will deprive the downstream tissue of oxygen and nutrients. Deprived of these, muscle cells cannot survive. Without oxygen, the myocytes will gradually die off, increasing the size of the infarction.
- 3) Presence or absence of collateral circulation. Occlusion of the main coronary artery deprives cardiac cells of oxygen and nutrients. However, the presence of a collateral coronary artery assists in containing the initial infarction area.

Generally, the larger the infarcted area, the greater the chance of death.

2.2 Treatment Options

There are currently no cures for heart failure, but there are treatments available to relieve the symptoms and to stop or slow down the progression of a failing heart. Using these treatments, HF patients can live longer and better.

life. Current heart failure treatments broadly consist of 1) medications, 2) surgical intervention and 3) changes in lifestyle. Heart failure is a serious condition that gets progressively worse over time. Recent reports of the reversal of HF condition with complete repair of the heart valve or controlling a fast heart rhythm can improve prognosis by preventing the further development of HF syndrome.

The types of treatment used to treat HF are highly dependent on the cause(s) of the HF. Medications are usually prescribed to manage underlying conditions, such as hypertension and diabetes. In more severe conditions, such as coronary artery occlusion or the presence of a heart failure, more invasive medical interventions are used. Typically, the treatment of HF patients involves a multifaceted approach, including the use of medications coupled with a cardiac device and/or surgical intervention to help the heart pump and contract properly. Although progress has been made in the treatment of heart failure, statistical data from NYHA demonstrate that the annual mortality rate remains high (5% to 20%).

The ultimate objectives of heart failure treatment are to

- 1) Improve the quality of life by relieving the symptoms
- 2) Attenuate the progression of the disease state
- 3) Prolong the life of the HF patients
- 4) Lessen the stress on public healthcare resources by reducing emergency room visits and hospitalisation.

2.2. *Pharmacological Approach*

The numerous medications used to treat heart disease can be divided into three main classes according to the following functions:

Dilate blood vessels

Angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor antagonists are types of dilators. These drugs aid the heart by dilating or relaxing blood vessels to improve blood flow and lower blood pressure, which helps the heart pump more effectively. However, approximately 10% to 20% of patients do not respond to ACE inhibitors. Examples of ACE inhibitors include enalapril, lisinopril and ramipril. Examples of angiotensin II receptor antagonists include losartan and valsartan.

Strengthen the heart's pumping action

Digoxin and beta-blockers assist in strengthening the heart's pumping action. Digoxin increases the strength of the heart's contractions, while beta-blockers slow down the heart rate. Both types of drugs tend to slow down the heartbeat and reduce blood pressure. Beta-blockers, such as carvedilol, metoprolol and bisoprolol, also help reduce the risk of abnormal heart rhythms.

Reduce water and sodium in the body

Diuretics, such as bumetanide and furosemide, help increase the excretion of water and sodium from the body. However, they can also cause potassium loss. Potassium-sparing diuretics, such as spironolactone and eplerenone, perform functions similar to diuretics but do not cause excessive filtration of potassium.

2.2. Device-Based Interventions

In cases where medication alone does not suffice, various surgical options can be performed to treat the underlying cause of heart failure.

The most commonly performed surgical options are angioplasty and coronary artery bypass surgery. Both of these surgical options target coronary artery disease. In angioplasty, a catheter is inserted into a blood vessel through a site in the groin or neck. The catheter is navigated to the site of the blockage and positioned at the site of the occlusion. A balloon mounted on the catheter is inflated, compressing the blockage materials against the wall of the artery. The procedure opens up the lumen, allowing the blood to flow freely. The compressed materials are removed. In some cases, a metal stent is inserted at the site to help maintain the lumen of the artery.

Figure 2.4: Illustration of Angioplasty
(Diagram taken from Boston Scientific, 2012)

In coronary bypass surgery, a blood vessel taken from other parts of the body, typically from the leg or chest, is used to bypass a blocked artery. This allows blood to flow freely through the blocked section of the artery.

Figure 2.5: Illustration of Coronary Artery Bypass
(Diagram taken from Mayo Clinic, 2012)

Other medical devices, such as heart pumps, left ventricular assist devices (LVADs), and implantable cardioverter-defibrillators (ICDs), can be implanted into the body to assist the pump function of a weakened heart. For Class III or IV patients, an ICD might be recommended. The function of an ICD is similar to a pacemaker. Apart from making shocks when it senses that the heart is beating abnormally, ICDs can also perform reconstruction surgeries, such as cardiac resynchronisation therapy (CRT), cardiac myoplasty, the Batista procedure, Dor procedure and

performed to ensure that the electrical signals that cause cardiac function as normally and as correctly as possible. The best treatment for patients is a heart transplant. Although there is typically a 50% survival rate for patients who receive a heart transplant, experience a significant improvement in survival and quality of life.

When heart failure is because of a congenital issue, such as a bicuspid aortic valve, intervention, such as heart valve repair or replacement, can be performed. In some situations, repairing or replacing the valve can correct the heart failure.

HF conditions are often attributable to a multitude of underlying conditions. Patients with diabetes have elevated glucose levels in the blood, which makes it difficult for the heart to pump efficiently. Also, elevated blood pressure increases the blood pressure, resulting in high blood pressure. In the case of a conglomeration of different disease conditions, the treatment for HF patients must be customised to the individual needs.

2.3 Natriuretic Peptides

Whereas the pharmacological approaches based on the drugs discussed in the previous sections have improved patient outcomes, mortality remain substantial. Currently available medications have specific functions, for example, ACE inhibitors and receptor blockers are used as vessel dilators but do not provide other benefits. Although drugs can help strengthen the pumping action of the heart, these drugs do not have a protective activity. Device interventions only partially relieve HF.

and do not provide any means for the heart to recover from improved and more innovative treatment options / strategies

2.3. *Cyclic Guanosine Monophosphate*

This second messenger, cyclic guanosine monophosphate (cGMP), is an essential component of signalling pathways that play a role in cardiovascular disease, including heart failure. cGMPs are produced through the action of highly specific guanylate cyclases (GCs) that convert guanosine triphosphate (GTP) to cGMP. The following three cGMP pathways are of particular importance to cardiovascular system:

- 1) Nitric oxide (NO)-sensitive GCs or soluble GCs: endogenous NO produced by endothelial cells binds to these soluble GCs
- 2) Natriuretic peptide receptor-A or NPR-A: NPRs bind to natriuretic peptides to produce cGMP
- 3) Natriuretic peptide receptor-BB: (NPR-B) receptors bind to C-type natriuretic peptides, which are secreted by endothelial cells

Figure 26: Illustration of Pathways Pertaining Cardiovascular System¹⁹

Cyclic GMP produced from these three pathways has various effector molecules, such as dependent on cGMP kinase (PKG), cGMP-regulated phosphodiesterase (PDEs), and 3) -regulated ion channels^[20, 21]. Consequently, these effectors lead to physiological changes, such as relaxing the blood vessel walls (vasodilation), increasing sodium filtration (natriuresis) in kidney (diuresis).

Importantly, although a common second messenger activates various physiological changes occur following cGMP production. Cyclic GMP produced by different NPs and their distribution will lead to different downstream effects. This is a differentiated cGMP signaling initiated by the distinct action profile of respective endogenous NPs. This topic will be discussed in detail in the next section.

The cGMP signal can be terminated by two mechanisms

- 1) Cyclic GMP hydrolysis into GMP through the action of PDE
- 2) Cyclic GMP leaches out of the cell into the intercellular space
of the leached cGMP is eventually eliminated through urinary excretion

2.3. Natriuretic Peptide Pathways

There are four types of natriuretic peptides (NP) in humans, ANP (type A NP), BNP (type B NP), CNP (type C NP) and urodilatin. ANP and BNP are produced by the atria and ventricles, respectively, while CNP is produced by endothelial cells. Urodilatin is a variant of the ANP precursor. The amino acid sequences of these endogenous peptides are presented in figure 2

Figure 27: Amino Acid Sequences of Endogenous Natriuretic Peptides

NPs are similar in their conformational structure and consist of a cyclic structure located within a ring structure. The ring structure is established by a disulfide bond between two cysteine amino acids. NPs are very specific

to their binding receptors. Following secretion by either endothelial cells, NPs circulate in the blood circulatory system and are located on the cell membrane. Both ANP and BNP bind to natriuretic peptide receptors, whereas CNP binds only to the natriuretic peptide receptor-2 [29]. Once NP binds to the complementary active site of the receptor, the cGMP second messenger is produced. As the elevation of cGMP levels within the cells leads to favourable changes in the body, which are beneficial to both the cardiac and

Although these peptides are structurally similar, they exert different physiological responses. Studies have demonstrated that treatment with ANP and BNP results in vasodilation, antihypertrophic, and lusitropic properties [30]. In contrast, treatment with CNP suppresses responses to ANP and BNP [31]. Other studies have demonstrated that ANP and BNP possess diuretic and diuretic functions, which are beneficial to HF conditions [32].

In contrast, CNP exhibits properties that are distinctly different from ANP and BNP. Unlike ANP and BNP, CNP exhibits pro-fibrotic and antifibrotic properties that are particularly important in the remodelling process of the heart [33]. Studies have shown that continuous infusion of the pro-fibrotic CNP in acute myocardial infarcted rats assisted in attenuating the infarction [34]. CNP also produces a less hypotensive state as compared to ANP and BNP [35]. In contrast to the vasodilating properties of ANP and BNP, CNP lacks the amino chain, which has been attributed

natriuretic function [40,41]. CNP such, although CNP is a good protecting agent, it does not exhibit properties.

2.3. Commercially approved NP

Currently, two exogenous NP (synthetic NP) and Nesiritide (synthetic NP), have been approved and are commercially available. Carperitide and Nesiritide are synthesized using the solid phase method.

Nesiritide

Nesiritide is a recombinant variant of human BNP, which was approved in 2001 for the treatment of acute decompensated HF. With a half-life of approximately 22 mins, Nesiritide exhibits a similar life of 18 mins. Exogenous peptide functions in a manner similar to endogenous peptide functions. Nesiritide was reported to exhibit similar properties and dilation with diuretic properties. Additionally, Nesiritide helps afterload reduction without increasing the heart rate. In addition to cardiac protective properties, Nesiritide also contributes to effect through its natriuretic function. Currently, Nesiritide is used in patients with acutely decompensated HF or patients experiencing

Several major clinical trials, such as the VMAc and NAPA FUSION, have evaluated the drug in different settings. The VMAc trial evaluated acute decompensated HF patients, while the NAPA FUSION trial evaluated chronic heart failure patients [42,43]. The NAPA trial, on the other hand, involved patients

bypass surgery in these patients. Nesiritide was reported as effective at the pulmonary wedge pressure, there was no evidence of beneficial effect [44, 47]. A possible reason for the lack of efficacy could be the treatment. This warrants for a chronic peptide delivery system. Choudhary published a feasibility study demonstrating that chronic subcutaneous infusion in both animals and humans was more efficacious [48, 49]. Currently, studies are going on to evaluate the efficacy of the chronic Nesiritide.

Carperitide

Carperitide, a recombinant variant of human ANP, was approved for the treatment of HF [50]. Japane reported that Carperitide is effective in reducing infarct size in acute myocardial infarcted patients. In an investigation of plasma creatine kinase levels and left ventricular ejection fraction, this finding was corroborated in a separate study. It demonstrated that treating acute myocardial infarction resulted in a decrease in the left ventricular end diastolic volume coupled with improved fractional [51]. However, currently, this peptide is approved for use

2.3. Designer Natriuretic Peptides

Given the complexity and diversity of HF conditions, it is not surprising that current commercially available ANP are not efficacious for every patient. Indeed, as discussed, Nesiritide is only effective at improving the hemodynamic condition and does not elicit the natriuretic property that has been demonstrated for human BNP. These have been designed

develop new NP with a improved action profile. Different NP variants have been synthesised by altering the peptide sequence to alter binding affinity and peptide susceptibility to enzymatic degradation.

Driven by the need for a better and more compelling NP drug, the University of Oxford and the University of Cambridge have engineered a novel chimeric natriuretic peptide. This peptide is a fusion of the amino-terminal 15 amino acids (AA) from human CNP and the C-terminal 15 amino acids from the *Dendroaspis* natriuretic peptide or DNP.

The novel NP, intuitively named CNP-DNP, was synthesised using a solid phase method on a peptide synthesiser, purified by reverse phase high performance liquid chromatography and validated by electrospray ionisation mass analysis. The amino acid sequence of CNP-DNP is shown in figure 2

Figure 28: Schematic Diagram of a Natriuretic Peptide

DNP was discovered in 1992 and was found to possess very potent diuretic properties. Functionally, DNP exhibits properties that are similar to ANP and BNP. All three peptides bind to NPR receptors but not NPR

receptor [13, 53], only CNP binds to the NPR. Of the three peptides that bind with the NPR, DNP demonstrates the highest affinity [53, 54]. The three peptides exhibit potent natriuretic and diuretic properties. In addition, it has been reported that DNP promotes cardiac unloading, but this is accompanied by significant hypotensive properties [55, 56].

Another key selection factor for the inclusion of DNP in the design was its resistance to degradation by neutral endopeptidase (NEP), which is its long-term minus. Indeed, the AA DNP minus is the longest of the known NPs; ANP and BNP minus measures 5 AA and 6 AA, respectively, and does not contain a terminal amino group. The length of the peptide is thought to provide resistance to degradation by NEP, hence its long-term stability is evident in CNP, which possesses a half-life of (2 minutes) of the NPs. The long half-life of DNP renders it an effective diuretic and natriuretic [48].

Following the fusion of DNP and CNP peptide sequences, studies have shown that this new functional peptide exhibits potent diuretic and natriuretic effects and minimal hypotensive effects with natriuretic and diuretic properties protecting against heart failure [16, 17].

CDNP, known commercially as Cenderitide, is currently in phase III clinical trials [11, 12]. *In vitro* studies have demonstrated that this design possesses a number of CNP properties, such as activation of cGMP in cardiac cells and antiproliferative actions. Furthermore, the half-life (approximately 10 minutes) is greatly improved compared with CNP [57].

Therefore, it was anticipated that this peptide would be effective of cardiorenal disease states, such as HF and AMI.

Indeed, the half-life of ANP, BNP and CNP are less than 2 min in humans, respectively. Given the short half-life and the low secretion levels, native peptides are typically ineffective at compensating for heart failure syndrome. Therefore, there is an interest in developing peptide delivery systems that maintain elevated plasma peptide levels for long periods.

2.3. NP Clearance

Native NPs are subjected to several inactivation mechanisms including receptor-mediated endocytosis, enzymatic hydrolysis (by dipeptidyl aminopeptidase, NEP) and renal excretion.

A third receptor, natriuretic peptide receptor 1 (NPR1), is known to bind to all of the NPs. This receptor acts as a clearance receptor for NPs. The amount of circulating NPs by sequestering them from the circulation. The half-life of NPs have been determined in rodents to be 5-10 min [58, 59].

2.4 In situ Polymer Precipitation System

In situ forming implant (ISFI) is a new class of controlled drug delivery system that has drawn much attention in recent years. Unlike traditional depot systems, these biocompatible systems do not require an invasive surgical implantation procedure. The formulation

relatively low viscosity, which allows for injection and makes the ISFI procedure less invasive and painful. The biodegradable systems also eliminates the need for a second procedure to remove the implant. In addition to drug delivery, ISFI has been investigated for use in three-dimensional cell culturing, cell transportation, and orthopedic administration [66].

Typically, following the injection of the ISFI subcutaneously into the tissues, the liquid system enters a biological environment leading to changes in the properties of the system. The properties, which are mechanical, lead to the formation of a drug depot. Another important feature of these systems is biodegradability. Biodegradation is a natural process that changes the properties of a polymer because of changes in the chemical structure. The breakdown of the polymeric matrix of the depot is useful in that gradual polymeric degradation of the depot allows entrapped drug to be released gradually over time. By carefully controlling the polymeric degradation, the duration of the drug release can be altered. Second, following degradation of the depot, the degradation products are removed via natural pathways, through either liver metabolism or kidney filtration. Therefore, there is no need for a second procedure to remove the implant.

In situ forming implants can be classified into three classes according to the mechanism of implant formation:

Figure 29: Overview of Forming Implant Technologies

1) *In situ* crosslinked polymer systems

These polymer systems make use of their surroundings to initiate crosslinking. Initiation of the crosslinking process is due to inherent physiochemical differences between the system and its surroundings or through an induced change [67].

2) *In situ* solidifying organogels

In situ solidifying organogel systems typically comprise amphiphilic lipids in a heated solvent. Following cooling, the solubility of the lipids or gelator starts to decrease. When the lipids aggregate to form a network, an implant is formed by a network of intermolecular physical interactions such as hydrogen bonds and van der Waals interactions [68].

Notably, organogels include glycerol fatty acid esters monooleate (GMO), glycerol monopalmitostearate (glycerolmonolinoleate).

3) *In situ* phase separation systems:

Because they are easy to formulate and systems that rely on this type of forming mechanism are the most popular classes. In this system, the gel interacts with the surrounding *in vivo* to initiate a phase separation process that results in a solidified depot [74]. ISFIs are suitable for both local and administration of substances with antimicrobial activity, hormones, substances interacting with growth factor system and so forth.

In this work, the system used was the precipitation drug delivery system, which is based on a solvent exchange, phase separation

2.4. Mechanism

Figure 2.10 Illustration of Polymer Precipitation Process

In solvent polymer precipitation drug delivery system is based on polymer precipitation through solvent exchange. This delivery system constitutes a biodegradable polymer in a biocompatible solvent dissolved or suspended. The resultant drug + polymer + solvent is administered by parenteral injection through a syringe needle. Injections are performed intramuscularly, intradermally or subcutaneously.

Following injection into an aqueous environment, the solvent from the gel solution interacts with the physiological surrounding, undergoing solvent exchange. In this process, the water solvent leaches out of the solution into the surrounding, while water from the surrounding tissues migrates gradually inwards. Once a critical water content is achieved in the gel system, the hydrophobic polymer begins polymer precipitation as a polymer phase inversion, and the critical water concentration is approximately 30% water content [29]. Phase inversion is a key step in the formation of the polymer matrix. The exact mechanism

gel solution responds to its surroundings determines the final morphology and the release characteristics of the gel depot.

2.4. Polymer Phase Inversion Dynamics

The manner in which the injected solution interacts with its surroundings affects the polymer phase inversion process. This, in turn, affects the morphological properties of the final gel depot, which in turn determine the drug release characteristics. Understanding the phase inversion dynamics and how the formulation parameters affect an effective injectable delivery system.

The two key fundamental parameters that directly affect the kinetics are as follows:

- 1) Liquid-liquid phase separation rate
- 2) Water influx rate in the gel

2.4.2 Liquid-Liquid Phase Separation Rate

Liquid-liquid (L-L) phase separation rate refers to the rate at which the gel solution effluxes out into the surrounding medium. This, in turn, affects the rate of polymer precipitation and the formation of a variety of structures.

Fast Gelling System

Systems that contain a hydrophilic solvent with a high water uptake, leading to water absorption and solvent efflux. This forms a fast gelling system.

by the addition of relatively hard polymer matrices with interporous macropores. The macropores in these systems are typically defined by their shape or long columnar structures.

Typically, drug release from a fast gelling system may involve an initial burst of drug release followed by a prolonged period of drug release. Polymeric macropores exhibit a relatively high drug diffusivity compared with the lower drug diffusivity in a polymer matrix [74, 75]. Therefore, during the initial drug burst release phase, the interconnected macropores make it preferable for a drug to diffuse out of the system. The drugs that are not released during the initial burst are usually entrapped in the polymer matrix. The low drug diffusivity in the matrix delays drug diffusion, and the release is expected to be delayed until the onset of polymeric degradation.

Figure 2.11 Comparison of Diffusional Path between Fast and Slow Gelling Systems

Slow Gelling System

In contrast, systems that consist of relatively hydrophobic surfaces with low water affinity demonstrate a slower water absorption and solvent uptake. These systems form a slow gelling system characterised by the slow and delayed drug release.

matrices with small isolated voids. The voids in these systems are not interconnected.

Drug release from a slow gelling system is typically characterized by an initial burst release followed by a uniform drug release profile. The smaller and non-interconnecting voids means that the drug release is controlled by diffusion through the polymer (of relatively low D), drug which results in the depressed initial burst release. Similar to fast gelling systems, following the initial burst release phase, entrapped drug gradually diffuses out of the depot. In this case, the pre-entrapped drug leads to a more uniform and consistent drug release. The release continues until the onset of matrix degradation and the remaining drug in the matrix are rapidly released.

Comparison of fast and slow gelling systems

As discussed, drug release characteristics are highly dependent on the gelling system used. In fast gelling systems, the drug release is controlled fundamentally by the phase inversion dynamics. This process involves the formation of interconnected structures through which the drug release occurs. Little drug transport occurs through the polymer-rich phase. In slow gelling systems, the drug release profile is less critically dependent on phase inversion dynamics than by the mass transfer kinetics. The non-interconnected small isolated pore structures means that the drugs have to diffuse through a continuous, polymer rich phase.

It should also be noted that whereas the initial drug release is strongly influenced by the system gelation time, the release characteristics are a function of the final depot morphology.

2.4.2 Water Influx Rate into the gel

Water influx rate refers to the migration of water from the polymer into the depot and the subsequent accumulation of water in the depot. The time and rate of water influx are crucial because a critical water concentration is required for the polymer phase inversion to take place. The critical water concentration is reached determines the type of phase inversion process and depot morphology, therefore, affecting the type of depot morphology. For a hydrophilic drug is of water influx rate is crucial because it determines the rate at which the drug dissolves and therefore the rate of drug release from the depot within and out of the implant.

The technique for measuring water influx rate was demonstrated previously by et al [19, 20]. Briefly, the water influx rate is quantified using a video imaging system. In this system, the gel solution is allowed to be in contact with a water reservoir. A video imaging system captures striations in the gel against a ground image. These striations represent interference fringes that are related to the distance the water has penetrated into the gel. The interpretation of the striations, as a function of the distance of water diffusion into the polymer solution is determined.

2.4. Parameters affecting Phase Inversion Dynamics

Since the inception of polymer precipitation delivery system, many parameters have been investigated, such as polymer concentration, polymer type, which were found to be relevant to understanding the dynamics are affected.

2.4.3 Effect of solvent

Polymer phase inversion dynamics are critically dependent on the solvent and water. Solvents with various water-miscibilities, such as pyrrolidone (NMP), dimethyl sulfoxide (DMSO), cyclohexyl triacetate [78, 83, 84], triethyl acetate [85], ethyl benzoate [77, 78, 86], benzyl benzoate (BB) [78, 87, 88], benzyl alcohol (BA), propylene glycol, acetone, tetrahydrofuran (THF), diethyl ether, ethyl acetate and low molecular weight PEG have been investigated extensively.

Hydrophilic solvents such as dimethyl sulfoxide (DMSO), pyrrolidone (NMP) and benzyl alcohol (BA) exhibit high affinity for water. The interaction between the solvent and water results in a rapid solvent efflux from the system and an increase in water absorption into the gel solution. This process occurs in the polymer precipitation delivery system, which rapidly solidifies polymer matrix.

Solvents such as benzyl benzoate, ethyl benzoate, triacetin, alcohols, are more hydrophobic and exhibit a larger miscibility with water. Therefore, these solvents tend to leach out of the system at a slower rate, which hinders water ingress, and the resulting polymer matrix is a semi-fluid polymer matrix.

These types of single solvent systems tend to provide drug release profiles that are often too extreme, exhibiting a release profile that is either too rapid or too slow. Newer strategies produce a more gradual and constant drug release profile by combining both hydrophilic and hydrophobic solvents into a single system. The creative manipulation of solvents with different hydrophilic and hydrophobic ratios allows drug release profile to be altered to the desired profile.

2.4.3 Effect of polymer type and concentration

The choice of polymer affects the speed at which gelation takes place. The type of polymer can be used in these systems, e.g. The biodegradable polymers include polylactide (PLA), copoly(lactide) (PLGA), poly(ϵ -caprolactone) (PCL) [34], polyhydroxyacids, polyanhydrides and polyorthoesters used as carriers. The PLA, PLGA and PCL polymers are usually used in their clinical practice [95]. Polymer hydrophilicity plays a critical role in promoting fast water ingress [76, 77, 79].

Generally, the polymer type and concentration affects the initial gel time. Typically, increasing the polymer concentration in the gel solution leads to a reduction in the initial gel time. Conventionally, a 4-6% polymer concentration is preferred. However, the higher the polymer concentration, the more viscous the gel solution becomes, which affects the casting process; therefore, a compromise must be made.

Studies have investigated the addition of a hydrophilic polymer to the formulation. One study demonstrated that the addition of a hydrophilic polymer (polyvinylpyrrolidone, PVP) drastically altered the initial drug release rate. The addition of PVP had no effect on the water influx rate, this has been shown to increase the release rate, which caused an increase in the initial burst release [107, 110].

2.4.3 Effect of Drug

Drug component in the gel formulation can also have a profound effect on drug absorption into the depot. Many different types of drugs have been studied, including forming polymer precipitation systems. These include small molecules such as lidocaine, aspirin, peptides, and proteins [78, 91]. The effect of the affinity of the drug at plays a critical role in the drug release [76, 77, 111]. Drugs that are hydrophilic in nature have been shown to increase the amount of water influx; influence is more pronounced in systems with lower water affinity.

2.4.3.4 Other notable effects

Other studies have investigated factors that influence the drug release rate, influx and drug release profile.

Effects of gel solution aging and release characteristics have been investigated. A gel solution that has undergone aging tends to form a more stable structure. Such structure formation with time of aging and

pronounced when a good solvent is used; however, the former structure is thermoreversible.

The effects of aqueous content in the gel solution has also been studied. When a small percentage of water is added to the formulation, it promotes a faster phase inversion process with inflexion. The presence of water in the gel formulation prevents it from solvent miscibility gap, which speeds up the phase separation and brings about the increase in the initial drug burst release.

2.4. Advantages and disadvantages of ISPP

2.4.4 Advantages

There are several advantages of using precipitation as a controlled drug delivery system. First, its injectability as the mode of administration offers greater ease of depot deployment compared with surgically implanted depot. However, careful consideration has to be taken when formulating with respect to gel viscosity. Second, a single gel injection can contain a high dosage. By altering the gel formulation, this can control the drug burst release and ensures a prolonged yet sustained release, effectively reduces the frequency of repeated dosing for chronic medications. This system not only promotes patient compliance and the quality of life. The manufacturing of the gel is relatively simple and the creative use of a biocompatible yet biodegradable host polymer as a delivery depot eliminates the need for a second surgical procedure to remove the implant.

2.4.4 Disadvantages

Although the advantages and simplicity of the precipitation delivery system have attracted much attention, a number of difficulties in the use of these systems.

The most widely debated problem is the solvent removal required for the removal of solvent for the polymer phase inversion to take place. The removal of solvent for the polymer phase inversion to take place into the physiological surroundings can potentially cause toxicity because of the presence of high amounts of organic solvents. This is even more compelling by the publication of toxicity studies [116, 117]. This will be discussed further on. Another problem is the reliability of the formation of the implant with respect to size. As discussed, the solvent exchange kinetics and the associated surface area strongly on the resulting implant surface area. By varying the size of the implant, this could alter the drug release kinetics. With a narrow therapeutic window, this issue should be considered carefully.

Finally, as with most drug delivery systems, it is critical to make sure that the organic solvents used might denature the more sensitive drugs. This significantly limits the type of drugs and applications suitable for use in this system.

2.5 Solvent

Solvents used in the polymer precipitation system come in contact with the drug in its physiological surroundings; therefore, only pharmaceutical grade solvents can be used. Commonly used solvents can be generally divided into two broad categories, hydrophilic and hydrophobic solvents. Hydrophilic solvents include dimethyl sulfoxide (DMSO), N-methyl-2-pyrrolidone (NMP), benzyl alcohol, propylene glycol, acetone, tetrahydrofuran. Hydrophobic solvents include benzyl benzoate, ethyl benzoate, triacetin, triethyl citrate.

In this study, two solvents were used, i.e., hydrophilic NMP and hydrophobic triacetin.

2.5.1 *N-Methyl-2-Pyrrolidone*

2.5.1.1 Physicochemical Properties

Of the hydrophilic solvents, NMP is the most popular organic solvent used in pharmaceutical formulations. NMP is a powerful, aprotic solvent with high solvency and low volatility. This colourless liquid has a high boiling point, high flash point and a mild odour. NMP is a small cyclic amide with the structural formula shown and exhibits high chemical and thermal stability.

Figure 2.13 Chemical Structure of NMP

NMP is also known by several other names such as *N-methyl-2-pyrrolidone*, *1-methyl-5-pyrrolidinone*, *1-methylazacyclopentanone*, *1-methylpyrrolidinone*, *1-methylpyrrolidone*, *pyrrolidin-2-one*, *1-methyl-2-pyrrolidinone*, and *2-methylpyrrolidone*.

NMP demonstrates excellent miscibility with water at all temperatures. It is a good cosolvent with many organic solvents, including alcohols, ketones, glycol ethers, and aromatic/chlorinated hydrocarbons. The solvent exhibits excellent solubility for a wide variety of other solvents, which makes it a suitable solvent for many applications. NMP is used extensively in petroleum, electronics, rubber and chemical polymer fields in various applications. Due to its excellent pharmaceutical solvency and biodegradable nature, it is increasingly used in pharmaceutical applications.

In pharmaceutical applications, NMP is widely used as follows:

- 1) As a solvent or preservative [12, 51, 21]
- 2) As a penetrator or enhancer [13, 14, 15]

2.5.1 Absorption, distribution, metabolism and excretion

Absorption: NMP is an excellent skin penetrator that is easily absorbed via the skin and gastrointestinal and respiratory tracts. The permeability rate of NMP is much higher than that of other commercial solvents [13].

Distribution: Following absorption, NMP is distributed in most tissues, with a high concentration in the sexual organs. The volume of distribution is 0.7 L/kg, and its plasma half-life is between 4 and 12 hours, depending on the mode of administration [13].

Metabolism: NMP is eliminated from the body through biotransformation into other compounds. As shown in Figure 2.14, NMP is readily hydroxylated to α -hydroxy-N-methyl-2-pyrrolidone (H-NMP), and in-H-NMP is oxidized to methylsuccinimide (MSI). MSI is further hydroxylated to α -hydroxy-N-methylsuccinimide (2H-MSI).

Figure 2.14 Illustration of NMP Metabolic Pathway [13]

Elimination: The main elimination route for NMP is through urinary excretion [13]. The elimination of NMPs is a safe process and unchanged NMP is directly absorbed by the body [13].

2.5.1 NMP Solvent Toxicity

NMP is listed in a number of pharmacopoeias. The US Food and Drug Administration (US FDA) approves a daily exposure of 5.30 mg/kg and classifies NMP as a solvent. NMP is also approved by the European Commission as a constituent of medical devices and is included in the list of additives in contact materials.

Despite extensive NMP toxicity studies performed including mice [14], rats [14], dogs [14] and humans [15], there are few studies documenting the NMP with respect to subcutaneous toxicity. Toxicity studies pertaining to subcutaneous or muscular toxicity are conflicting and contradicting data. For example, it was reported that NMP induced damage to skeletal tissue when injected intramuscularly into dogs [14]. It was concluded that these results may exhibit myotoxicity. However, another NMP biocompatible study in rhesus monkeys reported no tissue toxic responses, such as swelling, redness and irritation, were noted to be similar to the biodegradable polymers [15].

2.5.2 Triacetin

2.5.2 Physicochemical Properties

Figure 2.15 Chemical Structure of Triacetin

Triacetin is a triglyceride and is the triacetate ester of glycerol. It is a colourless, odourless, and tasteless liquid with a boiling point of 125.8°C. It is slightly miscible with water and is readily hydrolysed into acetic acid and glycerol. The half-life at pH 7 and 9 was reported to be 60.4 days and 16.5 days at 25°C. Triacetin is stable at pH 4.

Given its relatively safe toxicity profile, triacetin is used in a wide range of applications such as a flavouring agent and adjuvant, humectant, solvent and vegetable filters and teas, as a plasticiser; in the manufacturing of celluloid, photographic film, and as a fixative in perfumes.

In pharmaceutical applications, it is used as a hydrophilic plasticiser in polymeric coatings of tablets, capsules, beads, and granules, with

concentrations of 10% to 35% w/w. Triacetin is also used as antifungal drugs for the treatment of superficial fungal infections.

2.5.2 Absorption, distribution, metabolism and excretion

Absorption: Similar to the routes of exposure for NMP, triacetin is absorbed into the human body through skin and gastrointestinal and respiratory routes. Compared to other fats, triacetin is rapidly absorbed in the gastrointestinal tract.

Distribution: The distribution of triacetin following absorption is rapid; however, triacetin has been reported as a source of liver glycerol.

Metabolism: Similar to most fats, triacetin is readily hydrolysed to glycerol and acetic acid. Triacetin is also hydrolysed by human intestinal enzymes. It should be noted that triacetin hydrolysis is accelerated at pH 7-8.

Elimination: Glycerol is metabolized by the liver and adipose fat tissues as a metabolic pathway for storage. The elimination route for acetic acid is through the kidney through urinary excretion.

2.5.2 Triacetin Solvent Toxicity

The FDA has listed triacetin as a GRAS (generally regarded as safe) substance. A toxicity evaluation of triacetin was conducted by the Federation of American Societies for Experimental Biology (FASEB) demonstrated that triacetin was safe in long-term feeding and mutagenicity tests. In animal studies, a

toxicity were very low with no ~~of systemic toxicity~~ ^{of systemic toxicity}. In addition, human volunteers triacetin did not produce skin irritations or

2.6 Commercially Available ISPP System

Currently, three main drug delivery systems ~~is used as the~~ ^{is used as the} principle of separation, ~~namely~~ ^{namely} Atrigel® drug delivery system, Alzamer® ~~de~~ ^{de} and SabTM depot technology. Of these systems, only Atrigel® is available, ~~a FDA~~ ^{a FDA} approved product.

2.6. Atrigel® Drug Delivery System

The Atrigel® technology, developed ~~at by~~ ^{at by} ATRISX ~~based on a~~ ^{based on a} biodegradable PLA/PLGA polymer. This ~~is an~~ ^{is an} FDA approved product ~~is in plastic syringes~~ ^{is in plastic syringes} Atrigel® Delivery System & Syringe ~~with bipreuprolide acetate powder.~~ ^{with bipreuprolide acetate powder.} Both syringes are thoroughly mix by pushing contents of both ~~fro~~ ^{fro} motion for approximately 45 seconds to obtain a uniform suspension will appear colourless ~~and injected~~ ^{and injected} ~~intravascularly.~~ ^{intravascularly.}

The FDA approved products that utilise Atrigel® technology are

- 1) Eligard[®] Management of prostate cancer. This is a LHRH analogue (1209.4g/mol) controlled release formulation, designed to deliver a constant amount of testosterone in its blood. It is the most successful Atrigel product and is available in 3, 4 and 6 month doses [9, 10, 11, 13, 15]

- 2) Atridox® Treatment of chronic periodontitis. This is a subcutaneous release formulation which contains 48.45% (w/w) doxycycline released over a week period [156]
- 3) Atrisorb® Guided Tissue Regeneration (GTR) barrier product that does not contain any drug and is used for the guided regeneration of periodontal tissue [167]
- 4) Atrisorb AD product containing (44.44% w/w) doxycycline for periodontal tissue regeneration [168]
- 5) Doxyroba® product containing (44.44% w/w) doxycycline for the treatment of periodontal disease in companion animals [169]

2.6.2 Alzamer® Depot Technology

The Alzamer® depot technology was developed by the ALZA Corporation and is currently in preclinical stages. Although this system is also based on the PLA/PLGA polymer, the technology is unique in that it uses non-solvents, such as benzyl benzoate, rather than the highly hydrophilic solvents, such as the NMP used in Atrigel® systems. It is claimed that Alzamer® sustains the delivery of various therapeutic drugs, including proteins and other molecules for up to one month with minimal initial drug release [170].

2.6.3 SabertTM Depot Technology

The SabertTM depot technology system employs a high viscosity carrier such as sucrose acetate isobutyrate (SAIB), loaded with the drug [164]. Developed by DURECT Corporation, the SabertTM carrier forms a low viscosity solution when dissolved in organic solvents, such as ethyl acetate, and is used for the formulation of depot injections [171].

triacetin and propylene carbonate. The reported advantage of the Atrigel® system is the reduction in solvent usage. Typically, 50% NMP is used in the formulation, whereas for other systems, the solvent use can be reduced to between 15% and 20%. Products are undergoing clinical testing. Reliance Pharmaceuticals, which is currently in III clinical trials, is testing doxycycline (bupivacaine) developed for the surgical site. Administered during surgery at the surgical site, it provides constant local analgesia for 72 hours. Reliance was developed to treat schizophrenia or schizoaffective disorder, has recently been introduced.

Chapter 3 Materials and Experimental Methods

3.1 Materials and Reagents

High Performance Liquid Chromatography (HPLC) grade N-methyl-2-pyrrolidinone (NMP) and triacetin used as solvents were purchased from Sigma Scientific, respectively. Both solvents are of low toxicity and *in vivo* forming drug delivery systems. C-peptide drug was supplied by Therapeutics, Inc. A mixed buffer solution pH 7.4 was purchased from Sigma Scientific Pte. Ltd.

For the purpose of this work, the preferred delivery system was a sustained peptide release for approximately 1 month. Therefore, a biodegradable polymer was used. Low molecular weight poly(D,L-lactide-co-glycolic acid) 50/50, termed PLGA 50/50, was used as the matrix. The biodegradable and biocompatible PLGA 50/50 was purchased from BioMaterials (Gorinchem Netherlands). The number 50/50 follows the copolymer represents the D,L glycolide monomer molar ratio. The inherent viscosity of this random copolymer was 0.2 dl/g. For degradation, the PLGA 50/50 containing carboxylic acid as the

used. Addition of carboxylic end group will increase polymer hydrophilicity and hence the degradation profile.

3.2 Injectable Gel Synthesis

CDN P was first dissolved in NMP solution as Table 3.1 shows. The solution was stirred for at least 4 hours before the addition of polymer and triacetin solution. The polymer solution and drug concentration were prepared by weighing. The gel solution was allowed to homogenize overnight at room temperature. Fresh gel solutions were prepared 1 day in advance for all studies.

3.3 In vitro Release Study

3.3.1 Drug Release Study

The gel solutions were prepared the day before the start of the study. The day of the study, the gel solution was drawn into a 1 ml disposable syringe. Precautions were taken to ensure that no bubble was present in the syringe. A 0.2 cc solution was injected into 5 ml of buffer solutions (pH 7.4). The samples were placed in a rotator set at 150 rpm and incubated at 37°C. On each determined sampling day, the release medium was replaced with 5 ml of fresh buffer solution. The collected samples were tested for peptide concentration using a Biotek ELISA (Purchased from Thermo Scientific, Pierce Protein [Reference]). For the release study, which the eluted solvent quantification was not required, the collected released medium were used for quantification using HPLC.

Micro-BCA Protein Assay Kit

Peptide quantification was carried out using Micro BCA Protein Assay Kit. Micro BCA consists of a bicinchoninic acid formulation for colorimetric detection of total protein. Micro BCA was preferred over other assays such as the standard BCA protein assay and the modified Lowry protein assay, for its sensitivity at low protein concentrations. Micro BCA measures protein concentrations from 0.5 µg/ml up to a maximum concentration of 20 µg/ml. This kit also includes a calibration profile and a detailed protocol. Measurements of total protein concentration were performed using a UV-spectrophotometer (Shimadzu UV-2501).

Briefly, the Micro BCA kit consists of 3 reagents, which are mixed at a 1:1:1 ratio to formulate a working reagent. To measure the protein concentration, the sample and working reagents were mixed and were incubated at 37°C for 5 minutes. The samples were then cooled to room temperature before running on a spectrophotometer at 562 nm absorbance. A blank sample was run alongside the experimental samples to establish the baseline absorbance. The accuracy of the measurements of 7 peptide standards was measured at each release time point for equipment calibration.

3.3.3 Solvent Efflux Study

Solvent efflux quantification was determined using HPLC. The experimental conditions for solvent efflux study were based on the protocol discussed in section 3.2.1.

3.3.1. At predetermined time points, samples of the released medium were collected into glass vials, and the medium was replaced with fresh buffer. The released medium samples were filtered through a 0.22 µm HPLC sample grade filter. The prepared samples were transferred onto a HPLC sample vial and maintained at 4 °C.

The same HPLC system used for peptide stability study was used for the quantification. The operating conditions are as follows: 10 µl sample injection volume and a mobile phase consisting of 40% ACN and 60% water at a flow rate of 1.0 ml/min at a detection wavelength of 210 nm. The retention times of NMP and triacetin were expected to be around 4.6 and 3.6 min, respectively. Detailed HPLC working conditions are provided in Section 4.2.

3.3. Morphological Study

Both scanning electron microscopy and optical microscopy were used for morphological analysis and shell structure investigation. The shell structure is expected to affect the rate of gelation. As such, analysis of shell porosity is valuable to enable better understanding of gel formulation and its associated properties. Shell structure formation analysis will also aid in understanding the gelation mechanism from such system.

The gel solution was injected and allowed 3 days of immersion in water to form the outer shell structure to form a solid thick phase. The as-formed

was rinsed twice with deionised water to remove any salt residue from the surface. Following rinsing, the implant was frozen immediately for 10 min and fractured to expose the internal cross-section. The sectioned sample was lyophilised for 7 days in a laboratory freezer dryer (Telstar Industrial Ltd., Japan). The structure analysis was performed using scanning electron microscopy (SEM) (Hitachi Ltd., Japan).

For the optical microscopy images, a single drop of the gel was placed between a glass slide and cover slip. The cover slip was compressed against a thin gel sample. Water was introduced to make contact with the gel. Interaction observations were performed using a polarising optical microscope (Olympus Corporation, Japan).

3.3. Contact Angle Measurement

Hydrophilicity of the gel formulation is directly related to the contact angle. The lower the contact angle, the more hydrophilic the surface. The contact angle was measured using a First Ten Angstrom, FTA32. A small amount of gel was drawn into a syringe and attached to the equipment. The syringe plunger was precisely aligned with the mechanical pusher. The pusher was stopped when a drop of gel was dispensed from the needle tip and landed on the glass slide. Under the gel formulation on the glass slide, the contact angle was measured using FTA32. The measurement was repeated 10 times and the average contact angle was taken.

for that gel formulation. A detailed explanation of results will be discussed in Section 5.4

3.3.3 *Water Uptake Study*

The procedures for the water uptake study were performed based on the protocol discussed in section 3.3.1. At time points, the implant was removed from the buffer solution and was rinsed with deionised water to remove salt residue on the implant surface. Following the washing process, the water on the implant surface was blotted off using a laboratory Kimwipe and the implant weight was recorded. The water uptake percentage was calculated based on the following equation:

Where

W_{wet} = wet weight of gel at respective time points polymer,

W_{NMP} = weight of remaining NMP, weight of remaining triacetin

The equation considers the weight fluctuations as a result of solvent ingress and efflux. At each time point, water ingress, remaining NMP and triacetin in the implant together with polymer weight contributed to the total weight. The polymer weight was assumed to be constant throughout the first 24 hours of degradation. The buffer medium was tested for NMP and triacetin

efflux protocol. The solvent efflux quantification and calculation of the remaining solvent.

3.3. Degradation Study

The degradation study was performed in accordance with the described in section 3.3.1. At each time point, 3 gel samples removed from the sample. The removed samples were lyophilised using a freeze dryer before the samples were prepared for polymer analysis.

The lyophilised samples were dissolved in chloroform to produce a concentration of approximately 5 mg/ml. The dried samples were maintained in chloroform for 24 hours to ensure the complete dissolution of the polymer. After 24-hour dissolving process, the resultant PLGA/chloroform solution was injected into a 0.42m syringe fitted with an SEC sample

The polymer molecular weight analysis was performed using size exclusion chromatography (SEC) on a 1100 series SEC system (Agilent). The system consisted of an 1100 series solvent delivery system, a refractive index detector and a 300 mm x 7.8 mm ID, 5 μ m PL gel column. The operating conditions for the SEC setup were as follows: injection volume of 50 μ l, mobile phase consisting of 100% chloroform with a flow rate of 1.0 mL/min.

molecular weight calibration was established using a series of p ranging from 162 to 60,000 Da.

3.4 In vivo Release Study

3.4.1 Animal Care and Preparation

Ethics Statement

The *in vivo* studies were performed at CardioRenal Research Clinic (Rochester, MN). The Mayo Clinic Animal Care and Use *in vivo* study protocols (Mayo IACUC number: A34809

Male Wistar rats (Charles River Laboratories), weighing 150 were used for the study. The rats were maintained on a standard and were allowed 4 days to acclimatise to the animal facility at the start of each study. Throughout the entire study, the rats specialised *enrichment* ensure sufficient roaming space. The rats were timed with a 12-hour light cycle to promote a normal rodent activity

3.4.2 Blood Pressure Measurement

The rodent blood pressures were measured using the CODA pressure system for mice and rats (Kent Scientific Corporation). The system comprised a series of 6 cylindrical tubes in which the Tail cuffs were applied from the end of the tail up to the thick rats were acclimated to the internal environment of the cylinder

the rodents calm down, the blood pressure measurements were taken under minimal lighting. Once all 6 rats were calm enough, the measurement was initiated to begin the measurement process. In total, 30 measurements were taken for each rat at each setting. The measurements included the heart rate, systolic, diastolic and mean blood pressure. The rats that became agitated during the measurement process were removed, placed back in the rat housing to calm down before the measurements were retaken. The entire process continued until all of the blood pressures were measured successfully. Once the measurements were achieved, the rats were returned to their housings and their housings were returned to the rat housing.

3.4. Plasma Evaluation

The rodent blood samples were collected at pre-determined time points for the analysis of peptide and cGMP levels in the plasma. The blood was collected from the carotid artery, and the animals were sacrificed after the blood was collected. Following the collection procedure, the rodents were placed on a laboratory table and anaesthetised using 5% fluoroxene (in). The hairs on the neck were shaved to allow easy access to the underlying tissues. A small incision was made in the skin, exposing the subcutaneous fat layer. The fat layer, followed by the muscle, was teased away carefully to expose the carotid artery. A pre-determined length of PE tubing was inserted into the carotid artery and was tied down using a suture. The tubing was attached to the open end of the PE tubing. The measurement process was repeated until all the blood was collected, and the

dead once it stopped breathing. The collected blood was placed in EDTA tubes on ice. The tubes were centrifuged for 10 min at 12,500 rpm to separate the plasma and other blood constituents. The plasma was aliquoted and stored at -80°C until peptide or cGMP testing.

Radio Immunoassay of PK and cGMP

Radioimmuno assay (RIA) kits (Perkin Elmer Life Science, USA) were used to determine the CNP and cGMP concentrations. The CNP and cGMP RIA kit was used for CNP and cGMP quantification, respectively.

The CNP RIA assay is based on binding competition between the sample and a radioactive peptide found in the sample. In this case, the competition is between the sample CNP and ^{125}I -CNP. The limited quantity of antibodies specific for the CNP restricts the amount of binding sites. As the quantity of the peptide in the samples increased, the amount of ^{125}I -CNP peptide able to bind with the antibodies decreased. Quantification of the ^{125}I -CNP as a function of the peptide concentration was established using a gamma counter machine. In the cGMP assay, the function, the peptide concentration in both the plasma and urine was determined. The assay required overnight incubation. The cGMP RIA assay works on the same principle except that the peptide is replaced with appropriate analogs.

3.4. Urinary Output Evaluation

Urinary output was analysed for cAMP and cGMP excretion levels. The urinary output was evaluated based on a 24-hour metabolic cycle. The rats were maintained in individual metabolic cages in which the urine was funnelled into a collection bottle. Evaporation of the collected urine was minimised by maintaining the collection bottle on dry ice, which froze the collected urine. After the 24-hour collection cycle, the collected urine was thawed, measured for urinary output volume and analysed for cAMP and cGMP hormone analysis. The same RIA assays used to measure cAMP and cGMP in the blood were used to measure the cAMP and cGMP levels in the urine.

For each *in vivo* study, the baseline urinary output was collected from each rat. Subsequently, the 24-hour urinary output was collected before the blood collection day.

3.4. Statistical Analysis

All statistical analysis was performed using GraphPad Prism software. Data are expressed as mean $\pm$ SD, where data are expressed as mean $\pm$ SEM. Statistical comparison between groups by *t*-test with $P < 0.05$ was accepted as significant difference.

Chapter 4 *In vitro* Studies: Drug / Solvent Analysis

4.1 CDNP Analysis

4.1.1 CDNP Quantification

3 protein assay kits were selected to determine the best concentrations. These include Micro BCA Protein Assay Kit, Akitay BCA, and a Modified Low Protein Assay Kit. Several trials were conducted to find the best method for detecting NED. Ultimately, Micro BCA Protein Assay Kit was identified as the highest sensitivity and accuracy method for detecting peptide. A detailed explanation on the workings of this assay kit was described in the appendix. This method of quantification was used in all peptide release studies.

To further minimize any measurement error, standards were tested every peptide release point. Peptide standards ranged from 0.5ug/ml to 2.0ug/ml. Figure 4-1 shows the calibration plot for one of the calibration runs.

Figure 4.1: 0.5ug/ml to 20ug/ml CD-NP Calibration Plot

4.2 Solvent Analysis

4.2.1 Solvent Stability

A suitable method for solvent quantification has to be in place at the commencement of solvent stability calibration and solvent effluent analysis.

High Performance Liquid Chromatography (HPLC) was identified as an appropriate testing method and work was carried out to develop a suitable method that distinctly resolves both the solvents - acetonitrile and triacetin for quantification purposes. Below shows the final optimized HPLC method for the testing of NMP and triacetin.

HPLC Column Manufacturer: Agilent

HPLC Column: Zorbax 300S-B-18 Column, 4.6 x 250mm

Mobile Phase: ACN 40% H₂O

Run Time (min) 6.00

Detection wavelength (nm) 210

Figure 4.1 shows the peak definition of both NMP and triacetin. Retention time of NMP and triacetin was identified to be at approximately 3.6 mins and 3.8 mins respectively. The peak at 3.4 min is contributed by the buffer solution used to dissolve the solvents.

Figure 4.2: High Performance Liquid Chromatography Chromatogram illustrating dissolved NMP and triacetin peaks

With the final optimized HPLC method, solvent stability was investigated for its excellent thermal and chemical stability in a solvent system. Triacetin, on the other hand, is known to be stable in a pH 7.0 of water.

To investigate solvent stability in buffer solution, known [NMP] and [Triacetin] were prepared. Samples were rotated at 150rpm. The conditions were as such to mimic the study conditions. For 6 consecutive days, 1ml of prepared solutions was tested using HPLC for solvent concentration quantification. triplicates.

NMP Stability in Buffer

Figure 43 shows NMP chemical stability over a 6 day period. As expected, no changes in NMP concentration were observed throughout the study. This demonstrated that NMP is indeed very stable.

Figure 43: 6 day NMP Stability in Buffer [NMP] various

Triacetin Stability in Buffer

Figure 4-4 shows triacetin chemical stability over a 6 day period. Unlike NMR standards, triacetin concentration for all 3 known experimental concentrations drop over the entire test duration.

Figure 4-4: 6 days Triacetin Stability in Buffer [Triacetin]

Indeed, it has been reported that the half-life of triacetin is approximately 7 days in H_2O at pH 7.0. Triacetin is hydrolyzed to glycerol and acetic acid. changes in [triacetin] over a 6 day period could be calculated based on the half-life equation shown below:

Table 4 shows the calculated experimental triacetin concentration. At the given conditions (37°C incubation, rotation at 150 rpm), the half-life is averaged at 13.7 days.

[Triacetin] ug/ml	Calculated Half-life (days)
50	9.01 ± 20.3
150	8.7 ± 0.97
250	8.7 ± 0.75
Average	8.8 ± 1.10

Table 4.1: Calculated triacetin half-life based on 3 concentrations i.e. 50 / 150 / 250 ug/ml

The understanding of this half-life will assist in ensuring an accurate representation of data when conducting solvent efflux studies for triacetin.

4.2.3 Solvent Calibration

Following stability study, solvent calibration was established using the method described in 4.2.1.

NMP Calibration

Based on several publications, approximately 50% of the hydrophobic drug is expected to efflux out of the system within the first 24hrs. As such, the initial drug concentration could be estimated based on the following calculation:

Injected Gel Volume: 0.2cc

Total NMP loading: 80,000ug

Estimated 50% initial 40,000ug

Buffer Volume: 5ml

Estimated highest [NMP] 10,000ug/ml

A total of 18 standards ranging from 0.5ug/ml to 10,000ug/ml were used in the calibration run. 10,000ug/ml standard concentration should exist at this time. The lowest detectable concentration while lowest detectable concentration was found experimentally to be 0.5ug/ml. Calibration plot was based on the

Figure 45: NMP Calibration from 0.5ug/ml to 10,000ug/ml

Figure 54 shows the NMP calibration curve. It could be observed that the curve does not correlate linearly with NMP concentration. The curve follows a linear correlation between 0.5ug/ml and 10,000ug/ml.

In order to preserve a linear correlation, NMP calibration was determined by a range of 0.5ug/ml to 250ug/ml. Samples with expected NMP of which were determined to be 250ug/ml. the calibration Figure 46 shows the corrected NMP calibration.

Figure 46: NMP Calibration from 0.5ug/ml to 250ug/ml

Triacetin Calibration

As with NMP calibration, the calibration range was determined by triacetin burst release concentration and HPLC lowest detection

Injected Gel Volume: 0.2cc

Total triacetin loading: 40,000ug

Estimated 50% initial burst: 20,000ug

Buffer Volume: 5ml

Estimated highest [triacetin] in buffer: 4,000ug/ml

A total of 15 standards ranging from 5ug/ml to 10,000ug/ml were used in the calibration run. The lower limit of detection for triacetin is 5ug/ml.

Figure 4.7: Triacetin Calibration from 5ug/ml to 10,000ug/ml

Figure 4.7 shows the triacetin calibration curve. Unlike NMP, HPLC analysis of triacetin concentration is a very linear. The HPLC method used here has a lower detection limit of only 5ug/ml.

4.2.2 Effects of solvent

Following the quantification of the absorbance of triacetin in different solvents, the effects of solvents were investigated. Two main aspects were looked into: 1) concentration of triacetin and 2) solvent homogeneity and its influence on each solvent.

4.2.3 Concentration of solvent homogeneity

Solvent homogeneity is ~~critical~~ ^{critical} in ~~system~~ ^{system}. In an ideal ~~system~~ ^{system}, there be complete homogeneity ~~between~~ ^{between} ~~the~~ ^{the} ~~components~~ ^{components} of the system. This will allow drug and polymer to dissolve homogeneously within the solvent. Upon ~~turn~~ ^{turn}, the polymer will precipitate to form a polymer matrix where drugs are distributed throughout the entire ~~matrix~~ ^{matrix}. ~~It is also important to note that drug release is not only a function of the polymer matrix but also a function of the solvent composition and particle size and distribution between samples.~~

To test for ~~homogeneity~~ ^{homogeneity}, ~~two~~ ^{two} ~~different~~ ^{different} ~~solutions~~ ^{solutions} were prepared: 10% NMP / 20% TRI and 50% NMP / 10% TRI. ~~Immediately after vortexing~~ ^{Immediately after vortexing}, two samples were carefully prepared for each of the ~~solutions~~ ^{solutions}. One sample was collected from the solution nearer to the top half ~~of the solution~~ ^{of the solution} and the other was collected from the bottom half of the same solution. Extracted ~~via HPLC sample preparation protocol and tested on HPLC~~ ^{via HPLC sample preparation protocol and tested on HPLC}. The ~~entire~~ ^{entire} ~~solutions~~ ^{solutions} were ~~undisturbed~~ ^{undisturbed} at room temperature for 1 hour. After 1 hour, samples from the same collection locations were extracted and

Figure 48: NMP and Triacetin homogeneity in co-solvent system

Figure 4.8 shows NMP and triacetin results collected from this study. Both solvent solutions, the amount of measured NMP and triacetin were similar between samples analyzed at 0h and 1h. No significant difference was observed between samples collected from top half and bottom half of the vial. These observations strongly suggest that the two solvents were well mixed and no significant solvent partitioning was observed at the prepared solvent ratio.

4.2.3 Co-solvent Influence

After establishing that there is good distribution in the solution, another study was carried out to investigate the influence of the solvent system on the solvent partitioning.

In this study, solvent solutions were prepared such that there were two concentrations: 50ug/ml NMP and triacetin or 100ug/ml NMP and triacetin. Samples of 50ug/ml and 100ug/ml were prepared. Samples were rotated at 150rpm. At predetermined intervals: 0d / 1d / 2d / 3d, 1 ml was extracted from each sample and tested on HPLC for solvent quantification.

Figure 49: NMP and Triacetin influence in co-solvent solution

Figure 49 shows the results of solvent quantification. The bars represent the pure solvent samples while the bars with asterisks represent the co-solvent solutions. The asterisks indicate a significant difference ($p < 0.05$) between the pure solvent and the co-solvent solutions.

NMP Quantification

As discussed in section 4.2.1, the NMP in the initial and chemical standards was expected, the amount of NMP detected in MiP samples by HPLC was consistent throughout the 3 days. Interestingly, the amount of NMP in the solvent appeared slightly higher than the 50ug/ml and 100ug/ml standards. It appeared that there was some shielding effect from triacetin which reduced absorbance. This shielding effect appeared to be concentration dependent. For the 50ug/ml solvent sample, between 3 NMP detection varies by 5.36% to 10.92% compared to the control the variations for the solvent sampled from 0.7% to 7.15%.

However, it is worth noting that this reduction in NMP detected in triacetin is consistently observed in all known samples that vary. The difference between actual and expected concentration is typically

Triacetin Quantification

The trend observed with the pure compounds in the samples discussed in section 4.2.1. Triacetin half-life was found to be approximately 8.7 days experimentally, which is in line with the presence of triacetin. Triacetin was not affected by the presence of NMP. In all but 1 instance, triacetin concentrations in the solution were correctly quantified. This suggests that NMP has no influence on triacetin.

Chapter 5 *in vitro* Studies: Injectable Gel System Analysis

5.1 Drug Release Study

For the proposed application of this injectable gel system for peptide to treat heart failure in patients, it is considered that the system should exhibit a zero-order release profile over a course of 4 weeks, with little or

A huge initial peptide burst release is undesirable. Peptide also exhibits vasodilating properties. A sudden increase in plasma concentration could lead to drastic hypotension in patients, causing discomfort in the worst scenarios, and even death. Therefore, it was critical to avoid this by maintaining the initial burst release at a minimum. A minimum peptide release should be eliminated completely.

Peptide release from the gel system should exhibit a zero-order release profile. This is preferable for two obvious reasons. First, as with the initial burst release, a large peptide release at any point during the release is undesirable. Again, this will cause unnecessary discomfort to patients. Second, a

relatively ~~short~~ half-life of 18 mins. A rapid drug releasing profile result in unnecessary drug wastage. Peptide that ~~do not~~ bind to receptors will likely be deactivated by receptors and/or will be excreted by the kidneys.

Many factors contribute to the release profile of a gel system. Several different gel parameters, namely (1) polymer concentration, (2) solvent, (3) drug loading and (4) injected volume of the gel will influence the selection of the different parameters of the formulation considered. If a solution is too viscous, it will be difficult to syringe as ~~an~~ ~~stirring~~ application.

All constituent in gel formulations stated in the following section unless otherwise stated.

5.1. Effects of [Polymer]

Figure 5: *In vitro* study: Effect of Polymer Concentration

Polymer concentration will affect the release profile of this study. The gel formulations loaded with 0.15% peptide in varying amounts of polymer concentrations were used to investigate the effects of polymer concentration on the release profile. Figure 5 shows that the rate of release decreased with increasing polymer concentrations. There are two possible explanations for this.

First, the presence of a higher polymer concentration in the gel system slowed down the phase separation process. This is due to the fact that a higher polymer concentration results in a thicker gel network, which impedes drug diffusivity. Second, the presence of a higher polymer concentration in the gel system resulted in a slower water influx into the system, producing a relatively slower release profile.

gel system. As such, a reduction in the initial burst release phenomenon has been reported in several formulations of forming sy

Second, the initial peptide release could be ascribed to the s system. The dissolved could have eluted out of the system with t of the hydrophilic NMP solvent. In a gel formulation with a high the amount of NMP solvent in the system is lower. This means out of the system is reduced. Therefore, the amount of dissolved the system is lowered. Conversely, a formulation with a lower p contain a higher weight ratio of NMP. High level of dissolved the system together with the rapid NMP efflux results in a high initial release.

In the subsequent peptide release, the release rate for the sy concentration was much faster. In the 30% polymer concentration of the total drug was released within the first 18% was released in high polymer concentration formulation. As such, the of drug remaining in the depot for the former formulation was latter formulation lower amount of drug said 3 days resulted in a low concentration diffusional gradient, which explains the slower. Conversely, for the latter system, the presence of a higher re produced a higher drug diffusion and a lag phase in the release.

5.1.1 Effects of Solvent System

As discussed in section 2.4.3.1, the overall solvent hydrophilicity is a key factor in determining the system gelation rate. In this study, the effect of solvent system consisting of hydrophilic solvent (NMP) and hydrophobic solvent (PLGA) was investigated. This study was performed in two parts; the effect of solvent concentrations and polymer concentrations on the system was investigated.

5.1.2 Effect of Solvent

Figure 5.2: *In vitro* study: Effect of Triacetin Concentration

Figure 2.5 shows the release profiles of 0.15% peptide loading in varying NMP and triacetin concentrations. As expected, the gel formed in NMP as the only solvent exhibited the highest initial burst release. NMP is a hydrophilic solvent that rapidly demixes with water, leading to high efflu

rapid efflux promotes a fast phase inversion process and high resultant shell structure is one containing pores which are interconnected. This interconnected network of pores allows drug out of the system, leading to the high initial burst release.

Following the addition of 20% and 30% triacetin to the formulation, burst release was drastically reduced to $31 \pm 4\%$ and $13 \pm 4\%$, respectively. The hydrophobicity of the system was increased. The system hydrophobicity induced a slower phase inversion and resulted in a shell morphological transition from a high porous structure to a dense and nonporous. As explained, a slower gelling system results in small isolated pores within the shell structure. Consequently, drug migration through the polymer matrix, which exhibits inherently lower drug migration rate, leads to a reduction in burst release.

This study demonstrated that the addition of a small amount of triacetin, such as triacetin, produced a drastic drop in burst release. The results clearly demonstrate that triacetin helped to control the initial burst release.

5.1.2 Effects of [Polymer]

Figure 5-3: In vitro study: Effect of Triacetin and Polymer Concentration
 A) 20% Triacetin B) 30% Triacetin

Figure-5 shows the drug release profiles at various polymer concentrations in solvent system loaded with 20% (A) and 30% (B) triacetin.

Previous release studies have demonstrated that both polymer concentrations in solvent systems containing 20% triacetin demonstrated an average initial burst of $31 \pm 4\%$, whereas the systems containing 30% triacetin demonstrated an average initial burst of $13 \pm 4\%$. This result strongly suggests that shell morphological structures are determined by solvent hydrophilicity. Particularly in solvent system, the composition of hydrophilicity solvents will change the hydrophilicity. This will lead to the formation of different shell morphologies. To be effective for drug release in the formulation takes precedence only in fast gelling systems.

effects of solvent on thermic phase inversion are more significant than effects of polymer concentration.

5.1.2 Effects of [Drug loading]

Figure 5.4: In vitro study: Effect of Drug Loading
 20% Triacetin solvent system A1) % Cumulative Drug Release A2) Weight of Drug Release
 30% Triacetin solvent system B1) % Cumulative Drug Release B2) Weight of Drug Release

Figure 4.5 shows the release profiles of systems with varying drug concentrations. The plots demonstrated that the drug loading significantly affected the release profile. This was expected because the changes in the drug concentration relative to that of the polymer were small. The

another study in which a drug loading concentration ranging from 1 to 16% did not demonstrate an effect on the release profile. However, it has been reported that when the drug mass percentage is large enough, the structural phase inversion process is affected, resulting in a higher initial release rate.

5.1. Effect of Injected Volume

Figure 5.5: In vitro study: Effect of injected volume
A) Cumulative drug release B) Daily drug release

Figure 5.5 shows the release profiles of the formulation containing varying injected gel volumes. The injected volumes investigated in this study were 0.1 cc, 0.25 cc, and 0.5 cc. The initial burst release was similar for all 3 injected volumes, and size of the implant also influenced the drug release profile. In a larger gel surface area in contact with the surrounding solvent, the efflux out of the system is more rapid, and the influx into the system is slower. In this case, the effect of the differences in the gel surface area did not significantly affect the initial burst release, possibly because of the high drug concentration. Similar to the effects of polymer concentration, the effect of injected volume on the release profile was not significant.

influential role in a single solvent, fast solvent influx and efflux dominated the phase inversion process, masking its influence.

This study also demonstrated that the phase inversion process affects drug release. The phase inversion process played a significant role in the initial stages of drug release/solvent efflux. After the formation of the gel, release is based primarily on diffusion and is subsequently affected by polymer degradation. Referring back to the figure, compared with the larger injected volume (0.25 and 0.50 cc), the smaller injected volume (0.10 cc) exhibited a slower release rate starting from week 1. The larger surface area promotes faster polymer degradation, which was complemented by a faster peptide release.

5.2 Solvent Efflux Study

There are two key fundamental parameters that directly influence phase inversion kinetics. These are the phase separation rate and water influx rate. The *in vitro* results presented in the previous section demonstrated the importance of solvent in the phase inversion process. The solvent influx and efflux process helped control the shell structure and therefore the release profile. In addition, the *in vivo* release studies illustrated that the addition of triacetin altered the gel solution hydrophilicity and affected the release profile.

This section investigates and elucidates possible explanation drug release profile with respect to the gel composition discussed in this section.

Postulate 1: Initial peptide burst release is directly related to efflu
Postulate 2: Initial peptide burst release is directly related to the separation kinetics

5.2. NMP Efflux Peptide Release

Postulate 1:

Initial peptide burst release is directly related to efflu

This work demonstrated that phase inversion and water influx influencing the peptide release profile. However, directly, the relationship trend was observed suggesting that the increased initial peptide release associated with the presence of increased NMP in the gel formulation has a higher solubility in NMP, an increased in NMP content leads to a higher NMP efflux might result in the observed high initial release. In this section, whether peptide release is a direct consequence of the formulation was investigated. In total, 5 gel formulations containing NMP concentrations ranging from 20% to 60% in the formulation

loading were maintained at 0.45% and 40%, respectively. Ap
 triacetin were added to the formulation to make up 100% solvent

Figure 5.6: Drug vs. NMP Release Profile
 A) Daily Drug Release Profile B) Daily NMP Efflux Profile C) NMP vs.

Because only the initial peptide burst release was collected, only, on the first 3 days. At the respective time points, all of the bu from the sample bottle and was replaced with fresh buffer solu

solution was aliquoted into sample vials and stored until use. HPLC and drug content using micro BCA protein Assay Kit. Figure 5.5 initial day drug and NMP release plots, the peptide released was correlated with NMP solvent efflux amount.

Similar to the release studies, the peptide burst release at day 1 showed an upward trend with increasing NMP concentrations in the gel. The released peptide vs. NMP efflux plot clearly demonstrated that there was a clear trend of NMPs eluting together with the peptide. A correlation analysis showed that there is no significant correlation between NMP released and peptide released. Although data did not demonstrate conclusively that the peptide was eluted with NMP efflux, the data corroborated that the peptide burst was dominated by the dissolved peptide. The data also supports the postulate 1.

In addition, from Figure 5.6 it can be observed that although the gel for NMP released between 5,650,000ug, the corresponding burst release was much higher for single solvent system (9,500ug) as compared to the solvent systems (7,300ug and 5,700ug). This is not surprising as these systems have an effective initial burst release. This will be discussed in the

5.2. NMP vs. Triacetin Solvent Affinity

Postulate 2 states that initial peptide release is related to separation kinetics. This change in separation kinetics could be a result of solvent changes.

Subpostulate 2A:

Change in phase separation kinetics - solvent interaction of co

To understand further the solvent efflux mechanism, a study was conducted where there was solvent affinity between the postulated components. The postulate was that if NMP has a higher affinity for triacetin than for water, then the presence of triacetin in the solvent system would retard NMP efflux, slowing down the gelation process resulting in a more stable structure. In this study, 2 groups of gel solutions were prepared. One group contained 70% triacetin, whereas the other group contained 30% triacetin. Various concentrations of NMP were prepared and added into the formulation to make up to 100%.

Figure 57: NMP vs. Triacetin Release Profile
 A) Daily NMP Efflux Profile for 20% triacetin B) Daily NMP Efflux Profile for 30% triacetin
 C) NMP Efflux vs. NMP Loadings on Day 1

Figure 7.5A and B shows the daily NMP efflux. The trends observed in Figure 7.5A and B show that higher amount of NMP in the gel formulation were accompanied by higher NMP efflux on day 1. A plot of NMP efflux against NMP loading for day 1, as shown in Figure 7.5C, demonstrated that there is a significant correlation between the amount of NMP loading and NMP efflux. The red line represents the linear regression for both sets of data. The results illustrated that NMP efflux was solely dependent on the loading of NMP and not on the amount of triacetin, demonstrating that the presence of triacetin does not significantly change in NMP efflux amount. Hence disproving postulate 2A.

5.2. Overall Gel Hydrophilicity

Previous sections have demonstrated that efflux of NMP from directly affect peptide release. It also disproved postulation 2 rate of solvent system is not a result of retardation of NMP efflux. With these conclusions established, there is another possible change in phase separation kinetics. Solvent system in the overall gel hydrophilicity. This change in gel hydrophilicity could be a result of solvent efflux and water influx, leading to changes in shell structure.

Postulate 2B:

Change in phase separation kinetics is a result of changes in gel overall hydrophilicity.

Figure 5 illustrates two possible scenarios for the concentrations in a gel system.

Figure 58: Postulate of shell thickness to solvent concentration

In the first scenario, if both formulations contain the same different NMP and polymer concentrations, the formulation with should demonstrate a higher NMP flux. This should lead to a porous shell structure formation. If this is true, the peptide higher. However, this scenario contradicts the drug release that the amount of drug released was, this is not. Therefore unlikely to

In the second scenario, a faster efflux forms a thicker outer shell. In figure, the formulation with the higher NMP loading should form a more porous. However, given the thickness of the shell, the overall structure retards the rate of drug release. Therefore, both the higher and lower NMP formulations will exhibit a similar initial burst. In a separate study, the addition of 3% hydrophilic (PVP) polymer to the gel formulation led to an increase in the overall gel hydrophilicity. This resulted in a faster separation rate although no change in water influx. This was observed in the separation kinetics could have been attributed to the shell structure, as described in the second scenario.

To confirm this second scenario, morphological and hydrophilicity studies were performed. This is discussed in the following section.

5.3 Morphological Study

5.3.1 Gel sectioning

The formation of the outer shell starts almost instantaneously upon contact with the buffer solution. As the solvent exchange between the gel depot and surrounding medium occurs, the water precipitates the polymer to form the outer shell. Whereas the shell assumes structural integrity, the inner core of the gel depot remains in contact with the buffer solution. To mitigate the issue of a harder outer shell and a brittle implant, a fracturing technique was used to section the implant.

5.3. *Optical Scanning Electron Microscopy*

For the morphological study, 2 g of cross-linked gel prepared. Both gels comprised 20% triacetin and varying concentrations of PLGA. One gel contained 20% PLGA and 60% NMP, whereas the other contained 40% PLGA and 40% NMP. A third group, consisting of single solvent NMP gel, was prepared for a positive comparison for the shell structure and its porosity.

Figure 5.9 SEM / Optical-Cross-sectional Images of the 3 Gel Formulations

Figure 5.9 shows the cross-sectional images of the 3 gel formulations under electron and optical microscopy. As mentioned earlier on in Section 5.2.4, the PI solvent gel system, the shell morphology consisted of numerous cavities. These cavities were uniform in shape and covered the entire circumference of the section. These cavities exhibited high density.

with the cavities in the polymer. Consequently, for drug diffusion resistance pathways allowed on the surface of the peptide during the 24 hours. This evidence was consistent with the high peptide release in the release studies.

For the other gel formulations containing triacetin, the observed slightly different. As the NMP content decreased, the shell structure became more porous, highly interconnected pore structure to one that was denser structure. The smaller cavities made it more difficult for drug to diffuse through the low porosity. As a result, such, the initial drug burst release was suppressed. Although there were large cavities, these were significantly less in number and their shape.

Figure 5.10 Demarcation of the Shell Structure

Figure 5.11 Shell Thickness of Various Gel Formulations

* $P < 0.05$, Compared to control

Y $P < 0.05$, compared between fixed triacetin (TRI) groups

Figure 5 shows the demarcation of the shell thickness, and Figure 10 shows the shell thickness measurements based on the demarcation. Figure 10 shows that the shell structure was significantly thicker in the single NMP solvent gel system than in the solvent systems. With the addition of triacetin to the system, the overall hydrophilicity of the gel solution was enhanced. As a result, the system containing a large amount of NMP was more hydrophilic, leading to a thick shell structure.

The shell thickness data from the SEM analysis were consistent with postulation 2B (Figure 5.2.3). Postulation 2B suggests that hydrophilicity leads to separation in water, which, in turn, forms a thicker outer shell layer helped to impede or retard drug release. Therefore, in the shell thickness study, either the higher or lower NMP loading demonstrated similar initial release.

5.3. Water Uptake

The previous sections focused on the importance of phase inversion as mentioned, the hardening of the polymer on the water ingress rate was performed to demonstrate the difference in water uptake in solvent system and non-solvent system. In this study, the water uptake between the single solvent NMP 40% in NMP / 20% solvent in co-solvent system.

Figure 5.12 Water Uptake Profile for Single Solvent and System

Figure 5.12 shows a plot of the swelling increasing between the single solvent system and the co-solvent system. The figure shows that the single solvent system exhibited a faster and higher water uptake compared with the co-solvent system. The use of co-solvent reduced the overall porosity, which led to the slower ingress of water into the gel implants. Typically, the initial water uptake rate of the single solvent system is higher than that of the co-solvent system. The single solvent NMP system showed a sharp initial water uptake rate, which formed a number of sharp peaks, whereas the co-solvent system demonstrated a slower water uptake rate.

5.4 Contact Angle Analysis

Postulation 2B states that a change in phase separation kinetics can be observed in gel over very hydrophilic systems. Data from the morphological analysis of the gel systems is shown in Figure 5.13.

shell thickness demonstrated that the shell thickness decreased with the composition of hydrophobic solvent in the gel formulation. This postulation 2B. To further assess the validity of postulation 2B, the hydrophobicity of various gel formulations were analyzed. The hypothesis is that the ratio of hydrophilic and hydrophobic solvents in the gel formulation will affect the gel formation hydrophilicity. This change in overall gel hydrophilicity will affect the transition from a fast gelling system (hydrophilic NMP single solvent) to a slow gelling system (increasing amount of hydrophobic solvent triacetin). This resulted in a change in shell thickness and porosity. This is discussed in section 5.3.2.

Gel hydrophilicity was performed through contact angle measurement. As the gel formulation changes with its hydrophilicity, the contact angle will change. The higher the contact angle, the more hydrophobic the system is. This is due to a phenomenon known as wettability. A hydrophilic system tends to wet the surface more, resulting in a smaller contact angle.

In this contact angle study, 3 groups of gel formulations were analyzed. The formulations are as follows:

Group 1 40% Polymer / 60% NMP

Group 2 20% Polymer / 60% NMP / 20% Triacetin

Group 3 40% Polymer / 40% NMP / 20% Triacetin

Figure 5.13 Gel Hydrophilicity vs Shell Thickness of Various Gel Formulations

* $P < 0.05$, Compared with Control

** $P < 0.05$, compared between fixed triacetin (TRI) groups

Figure 13 shows the measured contact angle and the corresponding shell thickness for the 3 gel formulations. The figure demonstrated that gel formulations containing triacetin have a significantly larger contact angle as compared to the control group which does not contain triacetin. The two formulations consisting of the same amount of hydrophobic triacetin with varying amounts of PLGA and NMP showed a significant difference in contact angle and shell thickness.

demonstrated that a gel formed with a higher NMP composition has a larger angle than that with lower NMP composition despite both gels having the same amount of triacetin composition. This data strongly suggests that the formulation is more hydrophilic. This shows that the rate of gelation of the formulation is directly dependent on the overall gel hydrophilicity. This is consistent with the shell thickness result and further supports postulation that the gel formed with a higher NMP composition has a larger angle.

5.5 Polymer Degradation Study

The degradation profile of the polymer used in the gel system was compared between a cosolvent gel system (40% PLGA / 40% NMP / 20% triacetin) and a solvent NMP gel system performed in parallel.

Figure 5.14 Degradation Profile of Single Solvent Gel System

Figure 14.5 shows the degradation and polymer mass profile. As observed in cross-sections, the initial drug burst release was highly dependent on the gel structure. A fast gelling system produces very porous shell structure, leading to high initial drug burst release. However, following the hardening of the gel, the release rate decreases significantly.

the drug release rate shifted towards drug diffusion and its rate is also dependent on the rate of degradation of the polymer used.

PLGA 50:50, with an inherent viscosity of 0.2, polymer was coupled with the carboxylic end overall hydrophilicity increases. Consequently, the polymer attracts water and degrades faster. The polymer degrades faster than PLGA 50:50. The polymer chain is hydrolysed into acidic oligomers, which reduce the pH of the solution to approximately 4. In this case, the degradation profile did not differ significantly from that of the single NMP gel system.

The overall mass of the polymer matrix remained unchanged for 6 weeks before the polymer started leaching out of the depot, which gradually reduced the mass of the remaining polymer matrix. Following the loss of polymer, it was easier for the drug encapsulated within the matrix to diffuse out, as demonstrated by the drug release profiles shown in the previous chapter. Drug release typically started to increase after day 6.

Chapter 6 In vivo Studies

For the delivery of the ideal peptide release profile is one that order release profile in Ad , it is crucial to minimize burst release avoid inducing undesired hypotensive consequences. rate has to fall within a peptide therapeutic window in this case above $7 \times 10^{-7} \text{ kg/min}$. This dose has been demonstrated to exert beneficial effects in a myocardial infarction model.

Following the extensive studies, a gel formulation was selected based on the desired peptide release profile that was confirmed. This gel formulation consisted of 0.15% and 40% polymer dissolved in 40% NMP and 60% water. Figure 16 shows the peptide release profile and the daily peptide release from this system.

Figure 6-1: Peptide Release Profile of Gel Formulation
 A) % Cumulative Peptide Release Profile B) Daily Drug Release Profile

This gel formulation demonstrated NP release sustained for more than 30 days with an initial peptide burst of $11 \pm 1\%$ of NP eluting, which translated to $76 \pm 15\%$ of NP released within the first 24 hours. The rate of NP release started to decrease and assumed a linear release ranging from 5.95 to 0.1 mg/day over the next 30 days. Up to $50 \pm 1\%$ of NP loading was released by day 31.

In vivo studies (3 in total) were performed to evaluate the effect of the gel. The first 2 studies were performed in normal healthy rats, and the third was performed in the acute myocardial infarction rat model. This was because MI is the most common clinical representation among HF conditions.

6.1 Rat Study I (Normal Healthy Rats)

6.1. Study Outline

The purpose of this study in healthy rats was to compare the delivery of the peptide from the delivery-NF in D-threo-threo forming implant system. In this POC study, the formulation discussed above was used. Additionally, another formulation was used to elicit a release similar to that of the desired peptide release profile. The blood collection was performed at two time points: 24 hours and 2 weeks. The 24 hours time point was selected to determine the extent of release, whereas the 2 weeks time point was selected to determine the sustainability of this gel delivery system. The two gel formulations were:

Formulation 1: 0.45% ~~NP~~ / 40% PLGA 50:50 / 20% Triacetin in

Formulation 2: 0.45% ~~NP~~ / 40% PLGA 50:50 / 30% Triacetin in

In total, 25 male Wistar rats were randomly assigned into 5 groups as follows: Group 1, 24 hr treatment; Group 2, 2 week treatment; Group 3, 24 hr treatment; Group 4, 2 week F2 gel treatment; Group 5, vehicle group. The rats in this study were maintained on a standard diet and were allowed 4 days to acclimatise to the animal facility before the start of the study.

Gel Implantation

To achieve the required peptide dosage (40.2 μ g/day based on body weight), 0.7 cc of F1 gel was injected into each of the

whereas 1.0 cc of F2 gel was injected into each of the F2 gel group. The F1 gel group was injected with 0.7 cc of blank F1 gel.

To minimise the suffering of the animals during gel implantation, the animals were anaesthetised using isoflurane (1.5% in oxygen) and ventilated using a rodent ventilator. To minimise any unnecessary risk of infection, the animals (all of the rat) were shaved and swabbed with 70% ethanol prior to surgery. The respective volumes of freshly prepared gel were drawn into 20 G needles which were then attached to the syringe tip. The gel solution was administered via sequential subcutaneous injections into the rodents' backs. Following the injections, the rodents were returned to their cages and were allowed to recover.

6.1. Results and Discussion

6.1.2 Plasma Evaluation

Plasma cGMP concentration

Figure 6.2: In vivo rat study I: Plasma cGMP
A) Plasma cGMP (DB) Plasma cGMP

Figure 2.6A shows the results of the plasma concentration of NP (pg/ml) over time. The red line represents the NP concentration throughout the duration of release.

At 24 hours after the gel injection, the NP concentration was maintained at 115 pg/ml in the control group, which was similar to the background level. For the NP group, the NP concentration increased to $20,000 \pm 11,700$ pg/ml and $9,000 \pm 6,010$ pg/ml at 1 and 2 weeks, respectively. The NP concentration then dropped to $2,600 \pm 632$ pg/ml and $1,600 \pm 1,000$ pg/ml at 2 weeks after injection. The NP concentration decrease was observed because of the system's in vivo degradation. In 2 weeks, the NP concentration observed was somewhat unexpected. In an unpublished study by researchers at Mayo Clinic, the NP plasma concentration of approximately 5,000 pg/ml was observed for a dosage infusion rate of 100 µg/kg/day. *In vitro* data suggested that the NP released from the system would be higher than this targeted dosage. The NP concentration should be at least close to or higher than 5,000 pg/ml.

A slower rate of polymer degradation or a diffusion barrier within the tissue may have contributed to the lower NP concentration. In the physiological environment, the availability of water is limited compared with the *in vitro* environment. Because the degradation of the polymer is primarily due to the availability of water, the slower degradation might have contributed to the lower NP concentration.

slower release. In addition to exhibiting a lower degradation rate, week 2 results demonstrated that the injectable gel system provided protection against degradation of the drug and suggested that the structure was maintained. The assay is highly specific and detects only if the ring structure within the drug is intact. Therefore, week 2 suggested that the drug was intact and was in an active state.

Plasma cGMP Concentration

To investigate the bioactivity of the drug, the plasma cGMP concentration (pcGMP conc.) was measured. As discussed, cGMP is a second messenger activated through the binding of cAMP to A_{2A} and A_{2B} receptors. It has been demonstrated that only an intact ring structure is necessary for this activity [17]; therefore, the presence of cGMP would certainly confirm the release of the drug.

Figure 2.6B shows the plasma cGMP concentration (pcGMP conc.) for the control group, which represents the maximum pcGMP concentration in male Wistar rats, as reported by [18]. Likewise, the pcGMP conc. in the control group mirrored the pcGMP conc. in the treated groups. For the treated groups, the pcGMP conc. increased to 280 ± 76 pmol/ml for the 1 and 2 hr groups, respectively, after 24 hrs. After 48 hrs, the pcGMP level decreased to 170 ± 77 pmol/ml and 130 ± 39 pmol/ml for the 1 and 2 hr groups, respectively. The pcGMP concentrations for the treated groups were as expected. Given the finite amount of drug available, there is a maximum pcGMP concentration that can be achieved.

activation level. Therefore, despite the higher level at 24 h, the cGMP level remained at the maximum output level throughout the study. Although the differences were not statistically significant when compared, the absolute mean demonstrated an increase. The detection of increased cGMP in the NP group demonstrated the bioactivity of NP, the NP released and activated only when intact and bioactive with the ANP Receptors. Therefore, the NP must have retained its conformational structure.

6.1. Conclusion

This study demonstrated that the burst release from the gel was tolerated by the rodents. Additionally, peptide levels were maintained for up to 2 weeks. The lower than expected plasma measurements were due to slower polymer degradation. This study also demonstrated the peptide-protecting properties, which was evidence from using the detection of the conformational CNP RIA kit and 2) the increased cGMP level at week 2.

6.2 Rat Study II (Normal Rats)

6.2. Study Outline

This study served as an extension of rat study I. In rat study I, the gel produced the highest sustained release at week 2. Therefore, in this study, gel formulation 1 was used.

The release profile of any injected gel is dependent on the kinetics as well as on the rate of polymer matrix degradation. The degradation is dependent on intrinsic and extrinsic factors, one of which is because the polymer matrix degrades hydrolytically. Studies in a subcutaneous environment, the relatively limited availability of water, the polymer matrix tends to degrade at a slower rate compared with in vitro studies [67]. Polymer chain degrades hydrolytically. As such, lowered availability of water in a subcutaneous environment will retard rate of hydrolytic degradation. This phenomenon was observed in rat study I in which a lower peptide release was observed. Therefore, instead of injecting the entire gel volume into a single site, the gel was divided between several different sites, thereby reducing the initial burst. The smaller injection volume should provide a much larger surface area for degradation.

In addition to the multiple injection site strategy to improve peptide release, a smaller volume was used to produce a sustained release over a period of 3 weeks. Therefore, in study II, a gel volume of 2.8 cc was administered. Bearing in mind the initial burst effects from the initial burst release of the gel system, pre-injection was performed to separate the required 2.8 cc into 2 injections of 1.4 cc each. The injections were performed on day 0 and on day 2 to reduce the initial burst effect. A 4-week window in which the burst effect could dissipate.

In this study, male Wistar rats were randomly assigned into 4 groups as follows: Group 1, treatment; Group 2, treatment; Group 3, treatment and Group 4, vehicle group. The rats were maintained on a standard laboratory diet and were allowed to acclimate to the animal facility housing prior to the start of the study.

Gel Implantation

To minimise the suffering of the animals during the gel implantation, the rats were anaesthetised using isoflurane (1.5% in oxygen), and ventilated using a rodent ventilator. To minimise any unnecessary risk of infection, the back of the rat) were shaved and swabbed with 70% ethanol. Aliquots of freshly prepared gel (1.4 cc) were drawn into a 20 G needle and a needle was then attached to the syringe tip. Approximately 0.1 cc of gel was injected subcutaneously into each of 7 sites on the back of the rat. After the first injection, the injected gel was allowed to harden. After the injection, the rodents were returned to their cages and were allowed to recover. A second injection, an exact repeat of the first, was performed 48 hours later.

6.2. Results and Discussion

Without exception, the rats recovered well after each gel injection. No mortality took place within hours of the injection. A single rat from the vehicle group died after the second gel injection, and the cause of death may have been due to the same events that the rat experienced following the first injection. The

anaesthetisations on days 0 and 2 during the gel injection procedure. During the blood pressure injections, the rats were subjected to multiple blood pressure measurements. The procedure for blood pressure measurement involved holding the rat in a prone position. The spaceathwas heated to 37°C. This series of events may have contributed to the death of the rat that died.

6.2.2 Blood Pressure Measurement

As a noninvasive method to assess the presence of CDNP release in circulating rodents, the blood pressure was measured using a noninvasive method. The presence of any CDNP release was indicated by a lower blood pressure. Therefore, the blood pressure (BP) was monitored for both the control and CDNP groups. BP measurements were taken on days 0, 1, 2, and 3. Additional readings were taken within 24 hrs after each injection. Figure 6.1 shows the mean BP trend measured over the 18 days of the study for the control and CDNP groups respectively. Figure 6.2 and 6.3 shows the mean BP trend over the initial 3 days.

Figure 6.3: Blood Pressure Fluctuation over 18 days
 A1) BP trend for CD treatment groups A2) Mayg BP trend for treatment group
 B1) BP trend for vehicle B2) Magnified BP trend for vehicle group
 * $P < 0.05$, Comparison between BP of treatment and vehicle group at the

The BP trend for the vehicle group demonstrated a consistent 89.0 and 100.6 mmHg throughout. However, a number of drops in were observed during the first 3 days of the study, which was repeated exposure to isoflurane during the anaesthetisation process.

As expected, the mean CDN for the treatment group dropped from 102 ± 1.2 mmHg to 66 ± 1.7 mmHg within the first 6 hrs after gel injection.

coincided with the initial burst release from the gel system approximately 83 ± 1.8 mmHg within the first 24 hours. Following on day 2, the BP dropped from 86 ± 1.1 mmHg to 50 ± 3.0 mmHg, recovery from the initial burst effect of the second injection was maintained at between 76.7 mmHg and 90.9 mmHg until the end of the study. This decrease in the BP was significant compared with the vehicle group. The CDNP treated group confirmed that the CDNP was released continuously from the system over the entire 18 period and exerted its dilatory active function. A GNP infusion study in normal dogs also demonstrated a significant decrease in arterial pressure, and the CDNP release was significant.

6.2.2 Plasma Evaluation

Plasma NBP concentration

Plasma NBP concentration (pCNC) was quantified using the radioimmunoassay (RIA) kit. Figure 6.4 shows the results of the pCNC measurements, demonstrating the sustained release over the 18 study period. The measured NBP conc. was $33,000 \pm 12,800$ pg/ml, $13,02$ pg/ml and $8,011$ pg/ml at 1, 2 and 3 weeks, respectively. In the vehicle group, the measured NBP conc. was approximately 280 ± 160.4 pg/ml. The CDNP treated group clearly demonstrated an elevated pCNC that was significantly different from the vehicle group.

Figure 6-4: In vivo study Plasma cGMP
 A) Plasma cGMP concentration at 2 and 4 week time points;
 B) Plasma cGMP concentration at 2 and 4 week time points
 * $p < 0.05$, Treatment [173] Vehicle

If the *in vivo* data were representative of *in vitro* release, one would expect the NP conc. at week 3 time point to be similar to that at week 2. However, this was not the case. There are three possible explanations for this discrepancy.

First, the difference could be because there was a difference in the degradation of the polymer matrix compared to the degradation of the peptide. However, this did not appear to be the case. The slower peptide release was observed in the *in vitro* study, it was postulated that slower degradation was the reason. To confirm this, the gel injected in this study was separated into multiple injections. In a single mode of injection, it did not demonstrate an increase in NP 3 plasma concentrations at week 3.

Second, there could be NP bioactivity. As discussed, the CNP R1 receptor detects NPs with intact ring structures. As the PLGA matrix degrades, the NPs are released and can be detected by the CNP R1 receptor.

glycolic acids, it creates pockets of slightly acidic environment [17]. This internal acidic environment might have degraded the peptide. Because the RIA kit only detects intact cGMP, the resulting pcGMP conc. could be lower than expected.

Third, the gel implant was injected subcutaneously. Because it may dissipate from the site of the gel implant to all streamways at large portion of the residual peptide may have remained in the tissue, effect of peptide that entered the blood stream and therefore low concentration of the peptide. This was discussed further in chapter 7.

Plasma cGMP Concentration

The pcGMP conc. results showed an increase that the pcGMP conc. was 270 ± 41.4 pmol/ml, 190 ± 48.1 pmol/ml and $11,420 \pm 1538$ pmol/ml at 1, 2 and 4 week time points. The pcGMP vehicle group was approximately 100 pmol/ml. Although pcGMP conc. was elevated significantly at week 1 and 2, vehicle group, a less significant difference was observed at week 4.

The plasma cGMP concentration is dependent on the availability of cGMP. cGMP is produced by the activation of cGMP synthase by NO. Studies have shown that cGMP is dose dependent, and this relationship is at a high concentration. Based on unpublished data from previous studies (Burnett et al., 1992) the maximum pcGMP conc. in this particular rodent species is approximately 10,000 pmol/ml.

pmol/ml. It was also determined that a dose of 6,000 pg/ml is sufficient to achieve the maximum pcGMP conc. Given the high level of pcGMP, it was expected that the maximum activation of cGMP would be sustained over a 3-week period; however, this did not occur.

There is a possible explanation for this observation. In this pilot study, to achieve the sustained-release effect of the CDNP system; therefore, an CDNP was loaded into the alginate-chitosan hydrogel. The CDNP was loaded into the hydrogel at a high level, and a high level of pcGMP was achieved even at 3 weeks. The abundant pcGMP may have reflected the continuous maximal activation of cGMP over an extended period. This high level of pcGMP may not have been perceived by the animal. Therefore, compensatory responses might have intervened to regulate this abnormally high level of pcGMP. These responses will be discussed in detail in the following section.

6.2.2.3 Urinary Output Evaluation

Urinary Output Volume

Table 16 shows the urinary output for both the treated and vehicle groups. The mean 24-hour baseline urine output for the treated and vehicle groups was 13 ± 0.9 ml, respectively. After the first and second gel injections, the mean urinary output increased to 32 ± 2.5 ml and 32 ± 2.5 ml, respectively. The increase in urinary volume output was approximately two-fold. CDNP is known to be a diuretic; therefore, the increase in the urinary output directly after the injections may be attributed to the diuretic effect of CDNP.

during the first 24 hours after injection. This is further evidence that the released from the gel polymer matrix was biologically active.

Table 1: 24 hours Urinary Output Volume
(Baseline, 1st & 2nd injection, prior to sacrifice)

Urinary- cGMP and cGMP

The background urinary cGMP level, shown in figure 2 6 ± 0.10 pg/min and 4 ± 1.65 pg/min for treatment groups, respectively. For treatment group, 550 ± 139.60 pg/min and 600 ± 151.90 pg/min for the 1st and 2nd injections respectively. Immediately after the gel injection burst release of cGMP from the gel. Most of cGMP was excreted out of the system through urinary excretion, which accounted for the amount of cGMP excreted at 1, 2 and 3 weeks decreased to 107 ± 29.72 pg/min and 17 ± 8.19 pg/min, respectively. These are significant compared with the baseline levels. As mentioned, for the amount of cGMP released from the gel system was reduced to a m

level, which in turn led to NPO output. There was no significant difference in NPO output observed between the baseline and urine weeks for the vehicle group.

Figure 65: 24 hours Urinary CD-NP Output
A) Treatment groups B) Vehicle Group
* p < 0.05, treatment vs. Baseline

Figure 66: 24 hours Urinary cGMP Output
A) Treatment Groups B) Vehicle Group
* p < 0.05, treatment vs. Baseline

Figure 6.6 shows that the baseline urinary cGMP was 20 ± 0.10 pmol/min and 31 ± 2.68 pmol/min for treatment and vehicle groups, respectively. There was no significant change in the ucGMP output for the vehicle group after the injection of the blank gel. The ucGMP output increased significantly following the gel injections. The ucGMP output was 62 ± 3.32 pmol/min after the 1st and 2nd injection, respectively. The ucGMP output after the 1st injection was slightly lower than after the 2nd injection because there was a lapse time between the diffusion of the gel system into the blood, the binding with NAR/B receptors, the activation of the enzyme, and the filtration of pcGMP in the kidney. ucGMP output at weeks 1 and 2 was high. However, the output dropped to a level that was not significantly different from the vehicle treatment. An interesting observation was that the ucGMP output was high at weeks 1 and 2.

Earlier in this study, subcutaneous plasma cGMP and urinary cGMP were discussed. The gel system demonstrated an elevation of cGMP throughout the 3-week treatment period. The high plasma cGMP levels were consistent with the elevated cGMP activity seen in the kidney, as demonstrated by the initial elevation of the pcGMP output. The sustained hypotensive state of the treated rats.

However, in the morning pcGMP level did not maintain the saturation predicted by the level of ANP following the pcGMP saturation level. pcGMP decreased gradually over the next 2 weeks to a level different from baseline. Evidence from ucGMP excretion suggests pcGMP was not because of the loss of bioactivity. Despite the decreasing pcGMP level between week 1 and week 3, a constant was recorded for up to 2 weeks into the treatment. Instead, in the initial days of saturated cGMP production, the body may have an abnormal . Therefore, compensatory responses may have intervened to maintain an abnormally high level of circulating pcGMP. The two possible compensatory responses, both of which help reduce the circulating pcGMP, are as follows:

- 1) Regulating the rate of cGMP activation
- 2) Reducing the plasma cGMP level.

The first mechanism works by reducing the high cGMP activity. In this mechanism, the ANP receptor, the natriuretic peptide / cGMP pathway is regulated, preventing further cGMP production. The ANP receptor, also known as the NP clearance receptor, is a G-protein coupled receptor (GPCR) that binds ANP and BNP, initiating the removal of circulating cGMP, which helps to reduce the cGMP concentration and in turn, reduces the availability of cGMP for binding to ANP/BNP receptors. This increased pcGMP clearance was confirmed by the NP excretion data. In addition to the NP pathway, there is another cGMP pathway, the NO/cGMP pathway. This

pathway also promotes cGMP production and can be down regulated by the cGMP produced. [17, 41, 75]

The second mechanism involves reducing the circulating pcGMP achieved by increasing the cGMP removal rate through degradation by phosphodiesterases (PDE), such as PDE V, or more directly through

Based on the high sustained ucGMP output recorded at week 1, it was concluded that the circulating pcGMP conc. was lower than expected because of the high excretion rate. Therefore, this accounts for the gradual drop in pcGMP between week 1 and 3.

6.2. Conclusion

This study demonstrated that with the appropriate release formula, a sustained release of cGMP can be achieved for up to 3 weeks, although not at the level of the in vivo study. This study also demonstrated that a delivery system, it might not be necessary to sustain a high cGMP release. The key is to determine the right cGMP that is needed to stimulate a slightly elevated level of cGMP.

However, there were some limitations to this study. The bioactivity marker released was based on cGMP level. Antagonistic systems could be used in vivo setting; therefore, it might be necessary to at least have the

assess further the actual influence of these systems. Additionally, the gel system could be used in disease models to potentiate the effects of the drug.

6.3 Rat Study III (Diseased Model)

6.3.1 Study Outline

In addition to the predicate studies in normal healthy rats, the AMI model was used to investigate further the efficacy of the gel system because it provided a more clinical representation of HF conditions. In the predicate study, the volume of the AMI model was 2.8 cc. Over the 4-week period, the predicate study demonstrated that the volume of the AMI model could be reduced further. Therefore, for this AMI model rat study, the volume was reduced from 2.8 cc to 2.0 cc. The strategy to minimize the effects of the initial peptide burst release was continued in this model. The 2.0 cc targeted volume into 2 x 1.0 cc injections. The 1.0 cc injections were injected into 5 locations, with approximately 0.2 cc injected per site.

Male Wistar rats were assigned into 2 groups of 15 rats each. The groups were as follows: Group 1, vehicle group (gel only) and Group 2, AMI model (gel and CBNP). The rats used in this study were maintained on a standard diet and were allowed 7 days to acclimatise to the animal facility before the start of the study. In this study, 15 rats were used per group to account for the possibility of mortality sustained from the surgical procedure and the possibility of unsuccessful model creation. The study protocol involved injecting the gel solution on day 0, followed by the model creation surgical procedure on day 1.

finally, a second injection was administered on day 3. The rats were then returned to their cages for data collection.

Gel Implantation

Similar to the predicate study, the animals were anaesthetised (with isoflurane and oxygen) and ventilation was provided using a rodent ventilator. The animals were swabbed with 70% ethanol prior to the first set of injections. After the first set of injections, the rodents were returned to their cages and were allowed to recover. The second injection was administered using the same procedure at two different sites.

6.3.2 Acute Myocardial Infarction Model Creation

Following the first set of gel injections, the rats were retrieved for the acute myocardial infarction. Briefly, rats were anaesthetised as described in predicate study. The hair was shaved and the chest was swabbed with 70% ethanol. A 1.5 cm section of the chest wall was incised. The chest was opened using an intercostal incision and was retracted using a retractor. The left auricle was retracted slightly to expose the coronary artery system. Ligation, using a suture, was performed by tying the suture around the anterior descending coronary artery (LAD). The area immediately distal to the ligation was examined briefly to confirm the presence of ischemia. An ECG was administered over the heart to monitor the heart rhythm. The chest wall was closed using a 5/0 PDS blue polyester fibre suture with one layer through the skin and muscle. To minimise any inflammation, before suturing the suture.

skin, several drops of xylazine were added. The rats were removed from the ventilator and were kept warm using a heating pad. Buprenex was injected into the blood vessel to aid the recovery process.

6.3. Results and Discussion

In total, 30 rats were used in the study. However, 4 rats in the vehicle group did not survive the ligation surgery. All four died during the creation; the deaths resulted from heart failure during LAD ligation, massive internal bleeding or were due to the procedure. In addition to the 4 rats from the vehicle group (gel only) and 3 rats from the vehicle group (gel only) were excluded from the computation because of incomplete data. No deaths were recorded because of gelation. The data used in the discussion were based on 8 and 10 rats from vehicle and treatment groups, respectively.

Figure 6-7: Photo Showing the Injection Sites (Pituitary)

6.3.3 Blood Pressure Measurement

The predicate study performed by our group demonstrated that at a lowered level throughout the study, providing evidence that CD was eluted consistently out of the gelatin hydrogel. demonstrated bio

In this AMI model rat study, BP was measured before the start of sacrificing the rats. For additional data collection, BP was also measured after each injection to determine whether the BP dropped as a result of initial burst release.

Figure 8.6: Blood Pressure Measured at different time points
 *P < 0.05, Treatment vs. Vehicle of similar time point

Figure 8.6 shows the BP data collected at different time points. No change was observed in the vehicle group following the two gel injections. At the 3-week time point, BP had dropped from 100 ± 1.9 mmHg to 56.6 ± 0.8 mmHg. In the treatment group, BP dropped to 73 ± 1.1 mmHg and 69 ± 2.3 mmHg following the 1st and 2nd gel injections, respectively. The BP at 3 weeks was 79 ± 2.0 mmHg.

The following three observations show the BP measurement data:

First, in this study, BP of the treatment group was 73 ± 1.1 mmHg following the 1st and 2nd gel injections, respectively. Compared with the study in which the BP of the treatment group dropped to 50 ± 3.0 mmHg following the 1st and 2nd gel injections, respectively.

after the two injections, the drop in the BP of the study after was less dramatic. The observation of injection suggested that the rodents tolerated this depth and a more drastic drop observed in the predicate study. This difference in the BP drop was because of this study (1.0 cc per injection), which was lower than in the predicate study (1.5 cc per injection). As expected, the burst release was expected from the initial burst and this sudden elevation in the BP drop. The reduction in the amount of drug in the initial burst (due partly to the lower volume) resulted in an acceptable BP drop.

Second, the BP at 3 weeks in the treatment group was 79 ± 2 mmHg, which was a significant drop from the baseline BP level (compared to baseline) suggested that the gel was effective in reducing the BP even at 3 weeks. This observation was consistent with the results of the predicate study.

Third, the BP in the vehicle group remained unchanged following the infarction. However, as the infarction spread and worsened, the heart function deteriorated drastically, leading to a huge drop in BP from 100 ± 1.9 mmHg at baseline to 40 ± 1.9 mmHg at 3 weeks. Interestingly, the BP of treatment group at the same time point was 79 ± 2 mmHg, which was a significant drop from the baseline BP level, suggesting that the gel was effective in reducing the BP even at 3 weeks. Instead, the BP hovered around the baseline level, demonstrating further evidence of attenuation of the infarction in the treatment group.

6.3.3 Plasma Evaluation

The plasma NP concentration (pNP conc.) and cGMP concentration (pcGMP conc.) was quantified using the assay (RIA) kit. Data collected in Figure 6. The pNP conc. in the vehicle group was 120 ± 6.2 pg/ml and the treatment group was elevated significantly to $5,000 \pm 1,115$ pg/ml.

The pNP measurements from this rat study were consistent with predicate study. In the previous study, the administration of 2.0 cc of 505 pg/ml NP conc. at 3 weeks, which translated into approximately 1,115 pg/ml per 1 cc of gel. In this study, 2.0 cc of injected gel of 505 pg/ml per 1 cc of gel. In this study, 2.0 cc of injected gel of 505 pg/ml per 1 cc of gel. These data affirmed that the gel release was consistent between

Figure 6: Plasma Evaluation
 A) Plasma NP Concentration at 3 weeks
 B) Plasma cGMP Concentration at 3 weeks
 *P < 0.05, Treatment vs. Vehicle

The pcGMP conc. in vehicle group was 112 ± 8.0 pmol/ml, while baseline pcGMP conc. of 108 ± 15.5 pmol/ml in the normal rats group demonstrated a significantly elevated pcGMP conc. of 170 ± 21.4 pmol/ml.

weeks. This increased difference between the treatment demonstrated that the gel delivery system preserved CDNP for up to 3 weeks.

In the previous study, an excessive CDNP was injected into rats to demonstrate sustained release over a week period [17]. At the 3 week time point, the pcGMP concentration was reduced to a level that did not differ from baseline, despite the elevated pcGMP of $8,000 \pm 1,115$ pg/ml. It was hypothesised that the sustained delivery of high concentrations from the gel led to a consistently high cGMP activation rate. This high activation rate was recognised as abnormal by the body. Therefore, the drop in pcGMP was because of compensatory responses that intervened to regulate the amount of pcGMP. The primary compensatory responses identified were as follows: down regulation of NPR receptors to reduce cGMP production; stimulation of NPR receptor clearance may have helped to reduce the pcGMP level; therefore the reduction in pcGMP due to the high pcGMP level was lower than the increase in cGMP due to the high pcGMP level. This third mechanism was confirmed by the elevated urinary excretion of cGMP.

The plasma CDNP and cGMP data from this study supported the above postulation. In this study, at 3 weeks CDNP and cGMP concentrations were $5,000 \pm 1,115$ pg/ml and 170 ± 21.4 pmol/ml, respectively. In the previous study, the 3 week plasma CDNP and cGMP concentrations were $8,000 \pm 1,115$ pg/ml and 170 ± 21.4 pmol/ml, respectively.

pmol/ml, respectively. Comparing the two sets of CDNP, a high release (as demonstrated by NP release after 8,000 pg/mL) yielded a pcGMP conc., whereas CDNP release (this study) yielded pcGMP conc. at 3 weeks. In this case, because of the reduced dosage, cGMP activation was not as extensive, resulting in a higher pc

6.3.3 Urinary Output Evaluation

Figure 10 shows the CDNP and cGMP output at 3 weeks. The baseline vehicle group CDNP outputs were similar, 2.0 ± 0.23 pg/min and pg/min, respectively. As expected, the treatment group was significantly different at 120 ± 29.8 pg/min. This suggested that at 3 weeks, released from the gel was still in excess of the body needed.

Figure 10 24 hours Urinary Output
 A) CDNP Output B) cGMP Output
 *P < 0.05 vs. Baseline, P < 0.05 vs. vehicle

The baseline, vehicle and treatment group urinary cGMP outputs were 36 ± 2.2 pmol/min and 52 ± 4.1 pmol/min, respectively. In the treatment group, the cGMP outputs were elevated significantly above the baseline. Furthermore, the urinary cGMP output was significantly higher in the treatment group than in the vehicle group.

The elevated urinary excretion of cGMP suggested that the loading could potentially be reduced with this intervention, providing attenuation of the cardiac remodelling.

6.3.3.4 Cardiac Function and Structure Evaluation

Figure 6.11 Cardiac Function and Structure Analysis
 A) Ejection Fraction B) Heart / Body Weight Ratio C) LV Cardiac Fibrosis
 * $P < 0.05$, Treatment vs. Vehicle

Figure 12 Picrosirius Red Staining of Deposition

The cardiac function and structure of the vehicle and treatment groups were assessed. Figure-16. Left ventricular ejection fraction, assessed by echocardiogram, was significantly different at 43.1 ± 3.7% and 50.7 ± 3.7% in vehicle and treatment group, respectively. The heart / body weight ratio of the treatment group was also significantly different at 0.337 ± 0.005 and 0.357 ± 0.005, respectively. More important, a significant reduction in fibrosis was observed in the treatment group compared with the vehicle group. The fibrosis staining in vehicle group was 1.7 ± 0.3%, compared to 1.0 ± 0.3% in treatment group. Figure 12 shows the results of the picrosirius red staining.

Figure 6-13 Photo Showing Extent of Heart Infarctions

Figure 6-13 shows the extent of heart infarctions. The extent of infarction between treatment and vehicle group correlated with fractional area and heart / body weight ratio. Figure 6-14 shows the area of the heart infarcted area in the treatment group appeared, more superficially, than the vehicle group. The hearts exhibited a lesser extent of infarction. The percentage difference between the right and left wall of heart is measured at 89%. Treated group showed a 78% difference, while the vehicle group had the thinnest LV wall of 31% difference.

The deterioration of both cardiac hemodynamic function and associated with cardiac remodelling. In this case, the cardiac with the occlusion of LAD at the start of the study.

Data from the vehicle group demonstrated a marked decrease in from the reduced ejection fraction and hypotensive state expected in addition to the cardiac functions/body weight ratio, the presence of marked fibrosis and collagen deposition and the marked thickening demonstrated the remodelling mechanism in acute myocardial infarction. The CNP-treated group performed significantly better in all aspects of the analysis, demonstrating clearly protective effects of CNP. A recent study using chronic subcutaneous CNP in rats yielded similar results [17].

The potency of CNP for suppression of collagen production by inhibition of fibroblast proliferation [17]. However, CNP lacks the aldosterone-suppressing property, which is associated with ANP and aldosterone inhibitory property of ANP and BNP heart failure improves cardiac function, which was developed to attenuate the process in acute heart failure, exhibiting both the effects of CNP and RAS-suppressing properties of ANP / BNP. Therefore, the combined effect makes CNP an attractive peptide for attenuating the cardiac remodelling.

In this CNP delivery system, the conserved CNP released from the gel depot over the 3-week period demonstrated a distinct improvement in both functional and structural integrity of the heart. Both systolic and diastolic function showed better cardiac function, reflected by the improved ejection fraction. However, ejection fraction was not preserved completely, an improvement was demonstrated by the significantly higher BP compared to the vehicle at week 3. Structurally, the remodelling process was also demonstrated by the heart / body weight ratio in the treated rats, which was lower compared with the vehicle rats. This observation was also correlated with the reduction in fibrosis and collagen deposition.

6.3. Conclusion

The gel delivery system sustained CNP release over a 3-week period while maintaining peptide biological function and stability. The inflammation initiated with ligation, was attenuated, as indicated by the improved cardiac function in the CNP treatment group compared with the vehicle group. The subcutaneous injection allowed the system to mitigate the need for repeated intravenous administration of the therapeutic peptide. Intravascularly, the precipitation delivery system for the delivery of CNP is a novel and attractive approach for improving patient compliance and quality of life.

Chapter 7 In vitro In Vivo Correlation

7.1 IVIVC Classification

In this chapter, correlation between *in vitro* and *in vivo* data is discussed. *In vitro* *in vivo* correlation (IVIVC) is defined by the US FDA as a predictive relationship describing the relationship between the *in vitro* property of an oral dosage form and the *in vivo* response. Typically, *in vitro* property refers to the rate or extent of drug dissolution or release and *in vivo* response refers to the plasma drug concentration or amount absorbed.

The US FDA classifies IVIVC into 4 main levels. Description of correlation at different level of IVIVC is taken from FDA website.

Level A

This level of correlation is the best method of establishing the true IVIVC. Level A correlation is a point-to-point relationship between the *in vitro* and *in vivo* profiles. The establishment of a level A IVIVC allows the entire *in vivo* drug concentration-time course to be derived from the dissolution profile.

Level B

This level is generally considered less useful compared with Level B because this method is based primarily on statistical moment correlation. Level B correlation uses a summary parameter from the *in vivo* profile with a summary parameter from the *in vitro* profile.

Level C

This level of correlation establishes a correlation between a single *in vitro* parameter, such as AUC, and a single *in vivo* parameter, such as t_{max} . This level is generally considered the least accurate of the first 3 IVIVC level classifications. Level C correlation is preferred. In this multiple level C correlation, the correlation is established between several *in vitro* points and the mean of several *in vivo* parameters. The multiple level C correlation generally consists of at least 3 *in vitro* points, making it more accurate compared with the level C correlation.

Level D

This level of correlation is not considered a formal correlation. It is based on rank order and qualitative correlation and is generally not used for regulatory purposes. However, this level of correlation is widely used in the development of products in the conceptualisation stage. The Level D correlation serves as an aid in the development of a formulation or process.

7.2 Injectable Gel IVIVC

Because of the limited number of collection time points, a proper level IVIVC as described in sections 7.1.1 was not possible. In this case, a level D correlation was used.

A diffusion model was used to obtain a reasonable correlation between *in vitro* and *in vivo* data. To simplify the calculation and to keep within known parameters, a more accurate compartmental model was simplified to a single compartment model. The simplified model is shown in Figure 7.1.

Figure 7.1: Simplified Diffusion Model
Where V_d is Drug volume of distribution, C_{ss} is Drug Steady State Concentration

This single compartment diffusion model uses the drug diffusion coefficient, D , and the elimination rate constant, K_e . The plasma steady state concentration is represented by the following equation:

R is the amount of drug expected to be released from the system
 K_e is related by the following equation (assuming a zero order release):

In this case, the effects of drug transportation within the tissue and blood stream and the drug equilibration in the tissue were regarded.

In an unpublished infusion study performed by researchers at CDNP steady state concentration of 5,000 pg/ml was obtained with 43.2 $\mu\text{g}/\text{day}$. Using this result and the steady state equation, the CL_{DTP} value was calculated. CL_{DTP} (iv) value in Wistar rats was 8,640 ml/day .

Using this calculated value and the *in vitro* results, the expected plasma steady state drug concentrations for rat studies I, II and III. A compilation of the CDNP calculations is presented in Table 7.

Table 7-1: Comparison between Calculated and Measured Peptide Plasma-Steady Concentration (pg/ml)

The calculated plasma concentrations were based on the expected rate of release at the respective time points, whereas the measured concentrations were directly from the results.

With the exception of week 1 time point in rats, the measured values were either lower than the calculated values, the percentage difference between measured and calculated values was 1.82% and 3.84% at 24 hours and 2, respectively. Similarly, the other week 2 and week 3 time points were 100-300%. As mentioned previously, several factors, such as drug equilibration in the tissue, were neglected in the calculation. It is reasonable that the measured drug concentrations were lower than the calculated values.

Importance of the implant shape and size

In the *in vivo* studies, the shapes of the injected implants were generally spherical. However, the injected gels always result in a spherical implant because the gel was injected subcutaneously and the surface tension of the gel depress and spread out the gel during the shell structure formation process, the shape of the implants could differ. Therefore, in the 24 hr time point in rat study I, the measured drug concentrations were higher and closer to the calculated values compared with other time points, likely because of the high initial surface area, which promoted a rapid drug release during the initial 24 hrs.

Interestingly, the week 1 time point in rat study II also demonstrated a high drug concentration, which could simply be because of the implant not yet fully hardened. In the solvent gel system, it could have taken up to 1 week for the solvents in the implant were discharged. Consequently, the implant was in a semifluid condition, which allowed the peptide to diffuse out of the implant for a number of days following the initial 24 hours. As such, the drug release lasted for a number of days. In this study, in addition to the first set of injections, another set of injections were performed on day 7. The combination of drug release from the two sets of injections, coupled with the change in implant shape (described above), resulted in the higher than expected plasma

Effects of different injected volume

Using the data in table 7.1, a comparison between different injected gel volumes was made.

Rat study I differed from rat study II and III in terms of the injected volume. In study I, a single injection of 0.7 cc was administered, whereas in the other two studies, 0.2 cc injections were administered. At the week 2 time point in rat study I, the former exhibited a 183.24% difference, whereas the latter exhibited a 312.83% difference. In contrast, at the week 3 time point, the percentage difference was 100% in rat study II and III. This observation emphasised the importance of injected volume and the resultant drug release profile.

Figure 2: IVIVC Plot of Measured against Calculated CDNP Plasma Steady State Concentration
A) Inclusive of initial burst time point B) Exclusive of initial burst time point

Figure 2.7 shows plots of the measured plasma CDNP steady state concentration against the calculated values. As shown in Figure 2.7, the initial burst effect of the implant, i.e., at 24 hours and week 1. As discussed, these two time points show higher plasma concentration because of the change in implant structure at the onset of shell structure degradation in system. When these two time

were removed (Figure 7.1). Correlation analysis showed that there is correlation between measured and calculated plasma steady state concentration. The relationship followed a linear regression with a coefficient of 0.9923. In this regression equation, the ideal gradient is 1, with an equation of $y = 0.9923x$ indicates a linear relationship. The data demonstrated that the predicted concentration was typically 2.5% higher than the actual measured value. Certain differences between the *in vivo* measurements were expected, and the increased difference was largely because of the simplified single compartment model that was used. A multi-compartment model would certainly improve the accuracy of this relationship. However, the end points included in the studies did not allow for such detailed measurement. It was critical that these parameters be taken into consideration when calculating the expected C_{ss} . To achieve this, a pharmacokinetic study is recommended in future studies.

Nonetheless, the assumed IVIV relationship ($C_{ss} = C_p$) can verify some key points. First, the IVIV R^2 of 2.5X could be improved by using a multi-compartment model. Second, this diffusion model only holds for cases in which drug diffusion is the primary rate step. Third, to better model the initial burst, other models, such as Sigmoid, Weibull, Higuchi, Crowell, could be used, in which case, the collection time points should be taken together for the earlier phase of the

Chapter 8 Conclusion and Recommendations

8.1 Conclusion

8.1.1 *In vitro* Studies: *u/g*/Solvent Analysis

In this chapter, drug and solvent method of quantification was established. BSA protein assay was shown to be appropriate for quantification in buffer solution. Peptide concentrations ranging from 10 ug/ml to 20 ug/ml were demonstrated to form a linear relationship.

HPLC was used to quantify NMP and triacetin. A suitable HPLC method was established to resolve the buffer, NMP and triacetin peaks. The retention time of 3.66 mins and 4.642 mins, respectively. NMP was stable in buffer for up to 6 days. The linear relationship between HPLC absorbance and NMP concentration was established for NMP concentrations ranging from 5 ug/ml to 10,000 ug/ml; 5 ug/ml was the detection limit for triacetin. Triacetin, however, was shown to be unstable in buffer, and the linear relationship between triacetin concentration and HPLC absorbance was established for triacetin concentrations ranging from 5 ug/ml to 10,000 ug/ml; 5 ug/ml was the detection limit for triacetin.

Cosolvent homogeneity and stability was also investigated. NMP and triacetin exhibited good miscibility without significant solvent partitioning. The determination of triacetin concentration was independent of NMP concentration. However, the NMP concentration allowed to be expected in the presence of triacetin. 10% reduction was independent of triacetin concentration.

8.1. *In vivo* Studies: Injectable Gel System Analysis

In this chapter, the effects of various gel formulation parameters were investigated to assess their influence on the drug release profiles. Increasing polymer concentration in the gel formulation suppressed the initial burst release. This was likely due to the formation of a thicker and denser gel network, which impeded drug diffusion. In the addition of triacetin, the overall hydrophilicity of the gel solution, slowing down the release and suppressing the initial burst release. In the presence of varying concentrations, the hydrophilicity of the gel solution dominated the release profile regardless of polymer concentration. The suppression of the initial burst by the polymer concentration was magnified only in the single hydrophilic system. The effect of the volume was also masked by the dominating hydrophilicity of the gel system.

Solvent efflux was also investigated, and it was established that the hydrophilic NMP did not affect the amount of the injected drug.

NMP and triacetin solvents was also insignificant in this system. More important, the morphological analysis demonstrated that the structure changed with the overall hydrophilicity of the gel so that thickness increased when gel hydrophilicity increased. This postulation that a similar initial drug burst could be obtained at different hydrophilicities. The data from this study corroborated this postulation.

8.1. In vivo Studies

IN TOTAL, 3 IN VIVO RAT STUDIES WERE PERFORMED. THE FIRST STUDY IN HEALTHY NORMAL RATS ESTABLISHED THE TENDENCIES FOR THE SYSTEM. FIRST, STUDY ESTABLISHED PHARMACOLOGICALLY, RATS COULD WITHSTAND HYPOTENSIVE STATE INDUCED BY INITIAL DRUG BURST RELEASE. SECOND, THE PORE DENSIFICATION WAS SLOW IN THE SUBCUTANEOUS ENVIRONMENT COMPARED TO THE *VITRO* ENVIRONMENT. LAST, STUDY CORRELATED THE MAXIMUM CGMP ACTIVATION RATES AND PLASMA STEADY STATE CONCENTRATION AT 2 PMOL/ML.

The second normal rat study demonstrated that the bioactivity of the gel delivery system was preserved. This was demonstrated by the hypotensive state experienced by the group throughout the 3 test period. The presence of elevated plasma throughout the 3 weeks supported the preservation of peptide bioactivity. Despite the

throughout the 3 weeks, the cGMP activation rate was not significantly different from the control group. The study demonstrated an interesting yet compelling insight into the cGMP system in the body. cGMP was activated throughout the study, indicating that the high cGMP activation rate was determined by the rate of cGMP regulated the cGMP level by reducing the rate of cGMP activation and cGMP excretion rate. The sustained and elevated cGMP excretion rate was observed in the 24 hr urinary analysis.

The final study was performed in a rat model. The acute myocardial infarction model was created by ligating the LV at the start of the study, resulting in a hypotensive state and an elevated plasma peptide and cGMP levels in the treated group. The levels of cGMP were significantly different from the control vehicle group. The urinary excretion at 3 weeks demonstrated elevated cGMP and cGMP levels, suggesting further the possibility of developing a new treatment. Importantly, the distinct improvement in cardiac function and structural integrity of the heart were reported in the treated group. The cardiac function was characterized by the left ventricular ejection fraction, whereas cardiac structure was characterized by the heart/body weight ratio and fibrosis. The visual assessment of LV wall thickness was performed and was used to assess the extent of the infarcted area and the thinning of the LV wall. The data from the study suggested that the use of cGMP as a therapeutic approach for the treatment of heart failure and the delivery of the novel cGMP may improve both patient compliance and quality of life.

8.1. *In vitro* vs *in vivo* correlation

IN THE IVIVC ANALYSIS, CORRELATION WAS ESTABLISHED BETWEEN VITRO AND VIVO DATA. BECAUSE OF THE LIMITED NUMBER OF POINTS COLLECTED IN THESE STUDIES, A COMPLETE CURVE WAS NOT POSSIBLE. INSTEAD, THE RESULT WAS ESTABLISHED AS A SIMPLIFIED ONE-COMPARTMENT DIFFUSION MODEL. BASED ON THIS MODEL, THE PLASMA STEADY-STATE CONCENTRATION PREDICTED BY THIS MODEL WAS CONSISTENTLY 2.5X HIGHER THAN THE ACTUAL PLASMA PEPTIDE LEVEL MEASURED IN THESE STUDIES. THE OBSERVATIONS WERE AT 24 HOURS AND 1 WEEK POINTS. THIS OBSERVATION DEMONSTRATED THE FOLLOWING FINDINGS. FIRST, THE SIZE OF THE IMPLANTED GEL PLAYED AN IMPORTANT ROLE IN DETERMINING THE INITIAL DRUG BURST. THIS WAS ALSO TRUE DURING THE INITIAL FEW DAYS AS THE POLYMER DEPOSIT WAS UNDERGOING THE DEGRADATION PROCESS. SECOND, FOLLOWING THE DEGRADATION OF THE POLYMER, THE DRUG RELEASE CONTINUED TO BE CONSISTENT AFTER EQUILIBRIUM. THE 2.5X DIFFERENCE MAY HAVE BEEN BECAUSE OF PEPTIDE SATURATION IN TISSUE AND PERMEATION FROM IT INTO THE BLOODSTREAM. FACTORS THAT WERE NOT ACCOUNTED FOR IN THE SIMPLIFIED DIFFUSION MODEL.

8.2 Recommendations

8.2. Gel Ageing Study

In the reported *in vitro* studies, fresh gel solutions were always prepared a day in advance of the study to minimise variability in the age of the gel sample. From a commercial view point, it makes practical sense to study the stability of this gel solution and the ageing effect. The effect of ageing on the solution and its resultant release behaviour was investigated previously [10]. It was reported that polymer network formations typically form over time. These formations tend to increase over time. However, the formation of these structures was found to be thermoreversible. Upon heating or injection of the gel solution, the network formation disappeared. Therefore, it would be interesting to see how ageing affects the release in this particular gel system.

8.2. Detailed *in vitro* Correlation Study

A main limitation of the IVIVC discussed in this work was the lack of data points, especially during the earlier phase of the study. For a detailed IVIVC to be developed, a nonlinear model, such as Weibull or Hixson-Crowell models, must be designed to include collection times that are closer together, especially so for the first 24 hours. In the mathematical model, the gel samples and the tissue surrounding should be analysed for residual drug. This will provide a more accurate indication of the actual eluted peptide and the amount of residual peptide in the

With the depot residual *in vivo* drug release profile compared to the *in vitro* release profile plotted. This *in vitro* release profile will enable the time point comparisons between *in vitro* and *in vivo* studies. A widely adopted and profile comparison similarity factor f_2 , involving the calculation of similarity factors. These adaptations of the study will therefore ensure a correct IVIV correlation.

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