IN VITRO AND IN VIVO STUDIES OF
CONTROLLED RELEASE OF NOVEL PEPTIDE FROM IN SITU POLYMER PRECIPITATION DELIVERY SYSTEM

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# Table of Content

Acknowledgement ........................................................................................................ II

Table of Content .......................................................................................................... IV

Abstract ....................................................................................................................... IX

List of Figures ............................................................................................................... XI

List of Tables ................................................................................................................ XIV

Chapter 1 Introduction ............................................................................................... 1

1.1 PROJECT MOTIVATION ................................................................................. 1

1.2 PROBLEM STATEMENT .................................................................................. 3

1.3 OBJECTIVES AND SCOPE ........................................................................... 3

1.4 THESIS NOVELTY ............................................................................................ 5

Chapter 2 Literature Review ..................................................................................... 6

2.1 HEART FAILURE DISEASE STATE ............................................................... 6

2.1.1 Epidemiology ................................................................................................. 8

2.1.2 Etiology ......................................................................................................... 8

2.1.3 Acute Myocardial Infarction ........................................................................... 11

2.2 TREATMENT OPTIONS ................................................................................. 12

2.2.1 Pharmacological Approach .......................................................................... 14

2.2.2 Device-Based Interventions .......................................................................... 15

2.3 NATRIURETIC PEPTIDES .............................................................................. 17

2.3.1 Cyclic Guanosine Monophosphate ................................................................ 18

2.3.2 Natriuretic Peptide Pathways ....................................................................... 20

2.3.3 Commercially-approved NP .......................................................................... 22

2.3.4 Designer Natriuretic Peptides ....................................................................... 23
2.3.5 NP Clearance .................................................................................................................. 26
2.4 IN SITU POLYMER PRECIPITATION SYSTEM .................................................................. 26
  2.4.1 Mechanism ..................................................................................................................... 29
  2.4.2 Polymer Phase Inversion Dynamics .................................................................................. 31
    2.4.2.1 Liquid-Liquid Phase Separation Rate ............................................................... 31
    2.4.2.2 Water Influx Rate into the gel ............................................................................... 34
  2.4.3 Parameters affecting Phase Inversion Dynamics .............................................................. 34
    2.4.3.1 Effect of solvent ....................................................................................................... 35
    2.4.3.2 Effect of polymer type and concentration ............................................................ 36
    2.4.3.3 Effect of Drug ........................................................................................................... 37
    2.4.3.4 Other notable effects ............................................................................................... 37
  2.4.4 Advantages and disadvantages of ISPP .......................................................................... 38
    2.4.4.1 Advantages ............................................................................................................. 38
    2.4.4.2 Disadvantages ........................................................................................................ 39
2.5 SOLVENT ............................................................................................................................ 40
  2.5.1 N-Methyl-2-Pyrrolidone ............................................................................................... 40
    2.5.1.1 Physicochemical Properties ................................................................................. 40
    2.5.1.2 Absorption, distribution, metabolism and excretion properties ......................... 41
    2.5.1.3 NMP Solvent Toxicity ............................................................................................ 43
  2.5.2 Triacetin .......................................................................................................................... 43
    2.5.2.1 Physicochemical Properties ................................................................................... 44
    2.5.2.2 Absorption, distribution, metabolism and excretion properties ......................... 45
    2.5.2.3 Triacetin Solvent Toxicity ...................................................................................... 45
2.6 COMMERCIALLY AVAILABLE ISPP SYSTEM ................................................................... 46
  2.6.1 Atrigel® Drug Delivery System .................................................................................... 46
  2.6.2 Alzamer® Depot Technology ....................................................................................... 47
  2.6.3 Saber™ Depot Technology ............................................................................................ 47

CHAPTER 3 MATERIALS AND EXPERIMENTAL METHODS .................................................. 49
  3.1 MATERIALS AND REAGENTS ......................................................................................... 49
3.2 INJECTABLE GEL SYNTHESIS ............................................................................ 50

3.3 IN VITRO RELEASE STUDY .................................................................................. 50
   3.3.1 Drug Release Study ....................................................................................... 50
   3.3.2 Solvent Efflux Study ..................................................................................... 51
   3.3.3 Morphological Study .................................................................................... 52
   3.3.4 Contact Angle Measurement ....................................................................... 53
   3.3.5 Water Uptake Study .................................................................................... 54
   3.3.6 Degradation Study ....................................................................................... 55

3.3 IN VITRO RELEASE STUDY .................................................................................. 50
   3.4 CONTACT ANGLE MEASUREMENT .................................................................. 53

3.4 IN VIVO RELEASE STUDY .................................................................................. 56
   3.4.1 Animal Care and Preparation ...................................................................... 56
   3.4.2 Blood Pressure Measurement ...................................................................... 56
   3.4.3 Plasma Evaluation ....................................................................................... 57
   3.4.4 Urinary Output Evaluation .......................................................................... 58
   3.4.5 Statistics Analysis ....................................................................................... 59

CHAPTER 4 IN VITRO STUDIES: DRUG/SOLVENT ANALYSIS ................................. 60

4.1 CD-NP ANALYSIS ............................................................................................... 60
   4.1.1 CD-NP Quantification ................................................................................. 60

4.2 SOLVENT ANALYSIS ......................................................................................... 61
   4.2.1 Solvent Stability .......................................................................................... 61
   4.2.2 Solvent Calibration ...................................................................................... 65
   4.2.3 Effects of Co-solvent .................................................................................. 68
      4.2.3.1 Co-solvent homogeneity ..................................................................... 68
      4.2.3.2 Co-solvent Influence ......................................................................... 71

CHAPTER 5 IN VITRO STUDIES: INJECTABLE GEL SYSTEM ANALYSIS .......... 74

5.1 DRUG RELEASE STUDY ...................................................................................... 74
   5.1.1 Effects of [Polymer] ..................................................................................... 76
   5.1.2 Effects of Co-solvent System ...................................................................... 78
      5.1.2.1 Effects of [Co-Solvent] ....................................................................... 78
      5.1.2.2 Effects of [Polymer] ........................................................................... 79
6.3.3.4 Cardiac Function and Structure Evaluation ................................. 132
6.3.4 Conclusion ......................................................................................... 136

CHAPTER 7  IN VITRO - IN VIVO CORRELATION........................................ 137
7.1 IVIVC CLASSIFICATION .................................................................... 137
7.2 INJECTABLE GEL IVIVC ................................................................. 138

CHAPTER 8  CONCLUSION AND RECOMMENDATIONS .......................... 145
8.1 CONCLUSION ...................................................................................... 145
8.1.1 In vitro Studies: Drug/Solvent Analysis ........................................ 145
8.1.2 In vitro Studies: Injectable Gel System Analysis ............................ 146
8.1.3 In vivo Studies .................................................................................. 147
8.1.4 In vitro – In vivo Correlation ........................................................... 149
8.2 RECOMMENDATIONS ........................................................................ 149
8.2.1 Gel Ageing Study ............................................................................ 150
8.2.2 Detailed In vitro – In vivo Correlation Study .................................... 150

REFERENCE .......................................................................................... 152
Abstract

The *in situ* polymer precipitation delivery system has generated much interest over the last two decades. The ease of manufacturing and administering the delivery system are key advantages that have contributed to its increasing preference as a mode of drug delivery. Although much research has been performed on this system, the number of studies that have translated into animal models and subsequently into clinical trials is much lower than desired.

This study aimed to investigate and understand the release mechanism and kinetics involved in peptide release from an *in situ* polymer precipitation system. Moreover, this study aimed to establish a correlation between the *in vitro* and *in vivo*-derived data. The ultimate aim was to identify a delivery system that can be used to deliver a novel therapeutic peptide, CD-NP, for the treatment of heart failure conditions.

This study can be divided into 3 broad categories.

First, *in vitro* studies were performed to characterise systemically the delivery system and its efficacy at achieving the prolonged release of the drug Cenderitide. Various gel formulation parameters were tested, i.e., the effect of polymer concentration, co-solvent, drug-loading and injected volume. The co-solvent system using the gel formulation 40% PLGA / 40% NMP / 20% triacetin was the most suitable for achieving the desired linear peptide release profile. Investigation of the solvent efflux and its influence on the drug release profile and shell structure formation morphology were also investigated. The
solvent efflux and shell structure formation were found to be inter-dependent and affected the drug release profile.

Second, feasibility studies were performed on both healthy and diseased Wistar rats. As a preliminary feasibility study, the peptide release from an in situ polymer precipitation delivery system was performed on healthy rats. The results suggested that the delivery system was efficacious. In addition, the peptide bioactivity was preserved until the end of the prescribed study. The results from the diseased rat model correlated with the feasibility studies. The acute myocardial infarction rat model was created through left ventricular ligation. As expected, the preservation of peptide bioactivity was observed at the study endpoint. More important, in this disease model, the peptide delivery system effectively attenuated the heart failure syndrome, demonstrated by the distinct preservation of cardiac functionality and structural integrity.

Last, a simplified in vitro and in vivo correlation was established. Furthermore, it was established that the shape of the injected depot contributed to the initial peptide release. As such, a proper correlation between the in vitro and in vivo data during the solidification phase of the injected gel could not be established. However, following the complete solidification of the polymer depot, a steady peptide concentration was observed in the plasma. The estimated steady-state peptide plasma concentration was consistently 2.5x higher than that of the actual measured values. This phenomenon was attributed to the simplified single compartment diffusion model that was used to establish this relationship.
List of Figures

FIGURE 2-1: ILLUSTRATION OF THE TYPES OF HEART FAILURE ........................................ 8
FIGURE 2-2: ILLUSTRATION OF Atherosclerosis ................................................................. 9
FIGURE 2-3: ILLUSTRATION OF FORMATION OF ACUTE MYOCARDIAL INFARCTION .......... 11
FIGURE 2-4: ILLUSTRATION OF ANGIOPLASTY ................................................................... 15
FIGURE 2-5: ILLUSTRATION OF CORONARY ARtery BYPASS .......................................... 16
FIGURE 2-6: ILLUSTRATION OF cGMP PATHWAYS PERTAINING TO CARDIOVASCULAR SYSTEMS[19] .................................................................................................................. 19
FIGURE 2-7: AMINO ACID SEQUENCES OF ENDOGENOUS NATRIURETIC PEPTIDES[28] ..... 20
FIGURE 2-8: SCHEMATIC DIAGRAM OF CD-NP NATRIURETIC PEPTIDE[16] ....................... 24
FIGURE 2-9: OVERVIEW OF IN SITU FORMING IMPLANT TECHNOLOGIES[65] .................... 28
FIGURE 2-10: ILLUSTRATION OF IN SITU POLYMER PRECIPITATION PROCESS[73] .......... 30
FIGURE 2-11: COMPARISON OF DIFFUSIONAL PATH BETWEEN FAST AND SLOW GELLING SYSTEM[76] .................................................................................................................. 32
FIGURE 2-12: SOURCES OF VARIABILITY OF IMPLANT SHAPE AND SIZE[65] .................... 39
FIGURE 2-13: CHEMICAL STRUCTURE OF NMP ................................................................. 41
FIGURE 2-14: ILLUSTRATION OF NMP METABOLIC PATHWAY[139] .................................... 42
FIGURE 2-15: CHEMICAL STRUCTURE OF TRIACETIN ...................................................... 44
FIGURE 4-1: 0.5UG/ML – 20UG/ML CD-NP CALIBRATION PLOT ......................................... 61
FIGURE 4-2: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY CHROMATOGRAM ........ 62
FIGURE 4-3: 6-DAYS NMP STABILITY IN BUFFER AT VARIOUS [NMP] .............................. 63
FIGURE 4-4: 6-DAYS TRIACETIN STABILITY IN BUFFER AT VARIOUS [TRIACETIN] .......... 64
Figure 4-5: NMP Calibration from 0.5ug/ml - 10,000ug/ml ........................................ 66
Figure 4-6: NMP Calibration from 0.5ug/ml - 250ug/ml ........................................ 67
Figure 4-7: Triacetin Calibration from 5ug/ml - 10,000ug/ml .................................. 68
Figure 4-8: NMP and Triacetin Homogeneity in Co-solvent System ...................... 70
Figure 4-9: NMP and Triacetin Influence on each other in Co-solvent Solution .... 72
Figure 5-1: In vitro Study: Effect of Polymer Concentration .................................. 76
Figure 5-2: In vitro Study: Effect of Triacetin Concentration .................................. 78
Figure 5-3: In vitro Study: Effect of Triacetin and Polymer Concentration .......... 80
Figure 5-4: In vitro Study: Effect of Drug Loading ................................................. 81
Figure 5-5: In vitro Study: Effect of Injected Volume .............................................. 82
Figure 5-6: Drug vs. NMP Release Profile ............................................................. 85
Figure 5-7: NMP vs. Triacetin Release Profile ....................................................... 88
Figure 5-8: Postulate of Shell thickness relative to solvent concentration ........... 90
Figure 5-9 SEM / Optical Cross-Sectional Images of the 3 Gel Formulations ....... 93
Figure 5-10: Demarcation of the Shell Structure ..................................................... 94
Figure 5-11: Shell Thickness of Various Gel Formulations ..................................... 95
Figure 5-12: Water Uptake Profile for Single NMP and Co-solvent Gel System .. 97
Figure 5-13: Gel Hydrophilicity vs Shell thickness of Various Gel Formulations . 99
Figure 5-14: Degradation Profile of Single NMP and Co-solvent Gel System .... 101
Figure 6-1: Peptide Release Profile of In vivo Gel Formulation ............................. 104
Figure 6-2: In vivo Rat Study I: Plasma CD-NP & cGMP ...................................... 106
Figure 6-3: Blood Pressure Fluctuation over 18 Days ........................................... 113
Figure 6-4: In vivo Rat Study II: Plasma CD-NP & cGMP ...................................... 115
List of Figures

FIGURE 6-5: 24 HOURS URINARY CD-NP OUTPUT ............................................................. 119
FIGURE 6-6: 24 HOURS URINARY cGMP OUTPUT .............................................................. 119
FIGURE 6-7: PHOTO SHOWING THE 5x0.2-cc INJECTION SITES (POST-SACRIFICIAL) ........ 126
FIGURE 6-8: BLOOD PRESSURE MEASUREMENT AT PRE-DETERMINED TIME POINTS ....... 127
FIGURE 6-9: PLASMA EVALUATION .................................................................................. 129
FIGURE 6-10: 24 HOURS URINARY OUTPUT ...................................................................... 131
FIGURE 6-11: CARDIAC FUNCTION AND STRUCTURE ANALYSIS ...................................... 132
FIGURE 6-12: PICROSIRIUS RED STAINING FOR COLLAGEN DEPOSITION ....................... 133
FIGURE 6-13: PHOTO SHOWING EXTENT OF HEART INFARCTIONS ............................... 134
FIGURE 7-1: SIMPLIFIED DIFFUSION MODEL .................................................................. 139
FIGURE 7-2: IVIVC PLOT OF MEASURED AGAINST CALCULATED .............................. 143
### List of Tables

**TABLE 2-1:** Classification of Chronic Heart Failure .......................................................... 10

**TABLE 4-1:** Calculated triacetin half-lives based on 3 concentrations i.e. 50 / 150 / 250 ug/ml .............................................................................................................................. 65

**TABLE 6-1:** 24 Hours Urinary Output Volume ........................................................................ 118

**TABLE 7-1:** Comparison between Calculated and Measured ............................................ 141
Chapter 1  
Introduction

1.1 Project Motivation

Worldwide, cardiovascular diseases remain the leading cause of deaths [1]. Cardiovascular diseases come in various forms and usually end up with the syndrome of heart failure. Heart Failure (HF) is a condition in which the heart fails to supply sufficient blood flow to meet the needs of the body. Characteristics of HF include functional and structural changes in the heart, endothelial and vascular dysfunction with vasoconstriction, sodium and water retention by the kidney, and neurohumoral activation.

In United States alone, approximately 2% of the population or 5 million people suffer from HF, often known as congestive HF [1]. Patients with congestive HF account for about 1 million of the annual hospital admissions. These are patients who are admitted based on HF as a primary diagnosis. Another 2 million patients are admitted with heart failure as a secondary diagnosis. While most HF patients recover well from the reperfusion therapy received when in hospital, statistics show that a third of these patients get readmitted within 90 days after their discharge.

The increasing occurrence of HF is largely due to the lifestyle changes. Lifestyle such as over indulgence of oily foods, sedentary work life, lack of exercises, smoking are all contributing factors to the development of HF. HF failure conditions almost
always escalate on patients with pre-existing conditions such as hypertension, coronary artery disease, diabetes mellitus, family history of cardiomyopathy and obesity.

Indeed, with the increasing life expectancy coupled with the reluctance for a healthier lifestyle change, the occurrence of HF is most likely to further increase. Consequently, this will certainly add on additional stresses on the already overstretched public health resources.

Depending on the stages of HF, there is a variety of treatment options available for the patients. Generally, in the earlier stages of HF, treatments are more preventive-centric. Treatments such as long term oral medications and changes in lifestyle will usually be recommended to patient. For patients who are experiencing later stages of HF where medications alone are not possible to effectively control the syndrome of HF, surgical interventions may be called upon. However, once the patients enter into the later stages of HF, typically medication and surgical interventions will not be able to completely cure HF. In this case, early diagnosis and preventing the worsening of heart condition is still the best option.

There are various medications that can be utilized for the attenuation of HF conditions. One of the ways is to administer exogenous natriuretic peptides into HF patients. Natriuretic Peptides (NPs) are known to have beneficial effects in treating heart failure [2, 3]. Several synthetic versions of naturally-occurring NPs such as Carperitide (synthetic Atrial-NPs) and Nesiritide (synthetic B-type NP) have been approved for the treatment of acutely decompensated HF since 1995 and 2001.
respectively. The use of synthetic NPs, which models the naturally-occurring variants, has its limitations [4-7].

1.2 Problem Statement

Presently, administration of these peptides is done via infusion during in-patient treatment to control the HF conditions. Post-discharge, patients are required to perform frequent self-injections of these peptides as part of the long term control of the heart condition. However, these peptides typically have very short in vivo half-life of 4 - 20mins [8, 9]. As such, multiple peptide injection is needed on a daily basis. This greatly reduces the patients’ quality of life and is a potential source of reduced compliance. Statistics show that despite the improvements in HF conditions during in-patient hospitalization treatment, there is still a high occurrence of re-admission and mortality rate associated with discharged patients. It has been suggested that one possible reason for such high re-admission and mortality rate is due to compliance [10].

In order to improve on patient compliance, we should consider novel therapeutic strategies such as a peptide delivery system which could provide a sustained drug release over a longer duration. This delivery system should preserve the bioactivity and conformational stability of the peptide, protecting it also from enzymatic degradation while maintaining a sustained release over an extended duration. If achieved, this delivery system would have benefits such as improving patient 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 for acute HF, which is currently undergoing clinical phase III trials [11, 12]. This designer peptide combines the full native amino acid structure of C-type NP with 15-AA C-terminus of a green-mamba derived Dendroaspis NP peptide [13]. The resultant peptide, Cenderitide, possesses beneficial profiles from both peptides. It is shown that Cenderitide which acts on both Natriuretic Peptide Receptors (NPR-A and NPR-B), can avoid the hypotensive nature of B-type NP while the addition of the DNP moiety enables retention of the cardiorenal protective properties in congestive HF [14-17].

This thesis seeks to investigate on the possibility of formulating an in situ polymer precipitation delivery system for the sustained delivery of Cenderitide peptide. This work hypothesizes that a better control over the initial burst (low initial burst release) and subsequent peptide release profile can be achieved through manipulation of injectable gel formulation characteristics such as solvent content, drug loading and injected volume.

To verify the stated hypothesis, the work addressed the following objectives.

First objective: To study and understand the effect of various solution parameters on the controlled release of Cenderitide peptide an in situ precipitation delivery system.

Second objective: To identify one or more formulations that yield minimal burst, using in vitro studies.

Third objective: To validate the in vitro results on the selected formulation(s) in an in vivo model. This objective can be divided into two parts: peptide delivery system feasibility study in normal healthy rats and efficacy of delivery system in a diseased
model. The former serves as a proof of concept for the delivery system while the latter aims at elucidating preservation of peptide bioactivity through its effects on cardiac functions.

Fourth objective: To try to establish an *in vitro*–*in vivo* correlation for the *in situ* gelling system.

### 1.4 Thesis Novelty

Although the idea of *in situ* forming implants has been around for decades, very few studies have reported on controlled release of peptides. This work hopes to further the understanding of peptide release mechanism from such delivery system, in particular *in situ* forming polymer precipitation delivery system. Cenderitide is a very new peptide which is currently still undergoing clinical studies. The chief novelty of the work lies in the identification of key factors that minimize the burst release in a peptide-incorporated *in situ* gelling system. In this work we also show for the first time the *in vivo* effects of a sustained delivery system for Cenderitide, a novel natriuretic peptide used to control the effects of ventricular remodelling following a heart attack.
2.1 Heart Failure Disease State

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

The heart is very susceptible to damage from a variety of disease states. Under these circumstances, a healthy heart will begin to deteriorate gradually into HF syndrome. As the condition of the heart deteriorates, the body activates several neurohormonal pathways that release specific hormones. Osmoregulatory hormones, such as renin, aldosterone, and vasopressin, are released into the blood to increase the circulating blood volume and to reduce the blood pressure by relaxing the vascular wall. Whereas these hormones initially exhibit compensatory and beneficial effects on unloading the heart, prolonged circulation can have adverse effects, worsening the HF syndrome. For example, the prolonged circulation of renin and vasopressin leads to a decrease in renal perfusion. Consequently, sodium and water accumulate gradually in the body. Elevated aldosterone in the blood also leads
to sodium and water retention, and more critically, this hormone increases organ fibrosis, resulting in the worsening of cardiac remodelling.

If these conditions are unchecked, left ventricle (LV) remodelling occurs. In LV remodelling, myocyte hypertrophy and LV elongation occurs, enlarging the heart volume. This meant that although the stroke volume is increased, there is no actual increase in the ejection fraction (the pump efficacy). This is due to the lowered efficacy of the remodeled LV wall.

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

Many different causes and disease states that damage or overwork the heart muscle result in HF. HF can be broadly sub-categorised into systolic and diastolic HF. In systolic HF, the heart is incapable of performing a proper cardiac contraction efficiently enough to pump sufficient blood to all parts of the body. This situation leads to the insufficient delivery of the oxygen and nutrients needed for the body’s metabolism. In diastolic HF, the heart is unable to fill with sufficient blood during cardiac relaxation or experiences abnormal ventricular filling. Therefore, the amount of blood sent to the lung for oxygenation is decreased, resulting in an insufficient amount of oxygenated blood being diverted back to the left ventricle. Although 60%
of HF patients suffer from the former condition, there are also instances in which both conditions are present concurrently.

![Diagram of heart failure types](image)

**Figure 2-1: Illustration of the Types of Heart Failure**

### 2.1.1 Epidemiology

In the USA and most developed countries, HF is a common clinical outcome. In the United States alone, approximately 5.8 million patient or 2% of the population 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 HF patients identified each year and more than a million hospitalisations annually, HF is the leading cause of hospitalisation in elderly [1, 18].

### 2.1.2 Etiology

Many factors, such as congenital heart defects, accidental heart injuries and lifestyle, have been identified as causes of heart failure. The most common causes of heart
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, such as cholesterol, are deposited on the inner lumen of the coronary arteries. The gradual accumulation of these deposits, known as plaque, narrows the arterial lumen. This process, also known as atherosclerosis, affects the flow of oxygenated blood to the heart muscles. This diminished blood supply to the myocardial cells results in myocardial infarction.

![Figure 2-2: Illustration of Atherosclerosis](Diagram taken from National Heart Lung and Blood Institute, 2012)

Blood pressure is the measurement of the force acting against the arterial walls. In patients with persistent high blood pressure, this condition weakens the heart 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’s inability to produce enough insulin or utilise insulin properly. Persistent high blood glucose levels can
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 radiation and chemotherapy treatments for cancer, thyroid disorder, alcohol or drug abuse, HIV/AIDS and excess vitamin E. Common congenital heart defects that contribute to HF include cardiomyopathy, heart valve disease and arrhythmias.

The New York Heart Association (NYHA) published a classification table to assess the stage of heart failure in accordance with heart functionality. This table classifies the HF stages based on everyday activities and the patient’s quality of life.

<table>
<thead>
<tr>
<th>Class</th>
<th>Patient Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I (Mild)</td>
<td>No limitation of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, or dyspnea (shortness of breath).</td>
</tr>
<tr>
<td>Class II (Mild)</td>
<td>Slight limitation of physical activity. Comfortable at rest, but ordinary physical activity results in fatigue, palpitation, or dyspnea.</td>
</tr>
<tr>
<td>Class III (Moderate)</td>
<td>Marked limitation of physical activity. Comfortable at rest, but less than ordinary activity causes fatigue, palpitation, or dyspnea.</td>
</tr>
<tr>
<td>Class IV (Severe)</td>
<td>Unable to carry out any physical activity without discomfort. Symptoms of cardiac insufficiency at rest. If any physical activity is undertaken, discomfort is increased.</td>
</tr>
</tbody>
</table>

**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 management strategies are employed. For Class I patients, lifestyle changes are used to prevent the development of HF. For Class II patients, the indications of a structural heart.
disease require a strategy to prevent LV remodelling. In addition to lifestyle changes, medications are introduced in patients with Class III HF to help control the deterioration of HF syndrome. For patients diagnosed with Class IV HF, doctors will typically employ end-of-life care, high technology therapies or mechanical circulatory supports in addition to medication interventions.

2.1.3 Acute Myocardial Infarction
Acute myocardial infarction (AMI) is a common clinical outcome for various forms of cardiovascular diseases. AMI begins when myocardial ischemia occurs because of coronary artery occlusion. The limited or lack of oxygenated blood supply to part of the heart over an extended period leads to irreversible damage and death of myocardial cells. Typically, following myocardium necrosis from AMI, 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, 2012)
Of the main diseases causing HF syndrome, myocardial infarction (MI) 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 infarcted area depends on the following three key factors:

1) Occlusion location in the coronary artery. Generally, when the occlusion is located higher up in the main branch of the coronary artery, the affected infarcted area is larger.

2) Length of time of the occlusion. The longer the blockage is present in the coronary artery, the longer it will deprive the downstream myocardial cells of oxygen and nutrients. Deprived of these nutrients and, most important, oxygen, the myocytes will gradually die off, increasing the severity of the infarction.

3) Presence or absence of collateral circulation. Occlusion deprives downstream cardiac cells of oxygen and nutrients; however, the presence of a collateral artery assists in containing the initial infarction area.

Generally, the larger the extent of the MI is, the greater the chance of death.

2.2 Treatment Options

There are currently no cures for heart failure. However, various treatments are available to relieve the symptoms and to stop or slow down the gradual worsening of a failing heart. Using these treatments, HF patients can live a longer and more active
life. Current heart failure treatments can be categorised broadly as follows: 1) medications, 2) surgical intervention and 3) changes in lifestyle. In addition, although heart failure is a serious condition that gets progressively worse over time, there are reports of the reversal of HF conditions with treatment. For example, repairing a heart valve or controlling a fast heart rhythm can improve heart functionality, preventing the further development of HF syndrome.

The types of treatment used to treat HF are highly dependent on the underlying cause(s) of the HF condition. Medications are usually prescribed to patients with underlying conditions, such as hypertension and diabetes. In more severe conditions, such as coronary artery occlusion or the presence of irregular heartbeats, more invasive medical device-based treatments 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 to beat and contract properly. Although considerable progress has been made in the treatment of heart failure, statistical data from NYHA demonstrates that the overall annual mortality rate remains high (5% – 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 the need for emergency room visits and hospitalisation.
2.2.1 Pharmacological Approach

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

**Dilate blood vessels**

Angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers are types of vasodilators. These drugs aid the heart by dilating or widening the blood vessels to improve blood flow and lower blood pressure, which helps to unload the heart. However, approximately 10% to 20% of patients do not tolerate ACE inhibitors. Examples of ACE inhibitors include enalapril, lisinopril and captopril. Examples of angiotensin II receptor blockers are losartan and valsartan.

**Strengthen the heart’s pumping action**

Digoxin and beta-blockers assist in strengthening the heart’s pumping function. Both of these drugs tend to slow down the heartbeat and reduce blood pressure. Digoxin also increases the strength of the muscle contractions, which strengthens the heartbeat. Beta-blockers, such as carvedilol, metoprolol and bisoprolol, also reduce the risk of abnormal heart rhythms.

**Reduce water and sodium in the body**

Diuretics, such as bumetanide and furosemide, help increase fluid and sodium excretion and prevent water retention in the body. However, these drugs tend to promote the excretion of potassium and magnesium. Aldosterone antagonists, such as spironolactone and eplerenone, perform functions similar to diuretics without the excessive filtration of potassium.
2.2.2 Device-Based Interventions

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

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

![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, arm or chest, is used to bypass a blocked artery. The bypass allows blood to flow freely through the blocked section of the artery.

![Coronary Artery Bypass Graft](https://example.com/coronary_bypass.jpg)

**Figure 2-5: Illustration of Coronary Artery Bypass**
*(Diagram taken from MetroHealth System, 2012)*

Other medical devices, such as heart pumps, left ventricular assists (LVADs) and/or pace-makers, can be implanted into the body to assist the proper functioning of a weakened heart. For Class III or IV patients, an implantable cardioverter-defibrillator (ICD) might be recommended. The function of an ICD is similar to a pace-maker. Apart from pace-making, the device also monitors the heart rhythm and administers shocks when it senses that the heart is beating at a dangerous rhythm. Heart reconstruction surgeries, such as cardiac resynchronisation therapy, dynamic cardiomyoplasty, the Batista procedure, Dor procedure and Acorn procedure, are
performed to ensure that the electrical signals that cause contraction in the heart to function as normally and as correctly as possible. The best surgical option for HF patients is a heart transplant. Although there is typically a severe shortage of donor hearts, patients who receive a heart transplant experience a dramatic improvement in survival and quality of life.

When heart failure is because of a congenital issue, such as a heart defect, surgical intervention, such as heart valve repair or replacement, are options. In these situations, repairing or replacing the valve ensures correct heart function.

HF conditions are often attributable to a multitude of underlying disease conditions. Patients with diabetes have elevated glucose levels in the blood stream, which makes it difficult for the heart to pump efficiently. Elevated glucose levels also increase the blood pressure, resulting in high blood pressure condition. Given this conglomeration of different disease conditions, the treatment options for individual HF patients must be customised to the individual needs.

2.3 Natriuretic Peptides

Whereas the pharmacological approaches and medical device-based interventions discussed in the previous sections have improved patient outcomes, morbidity and mortality remain substantial. Currently available medications often have very specific functions. For example, ACE inhibitors and receptor blockers are effective vessel dilators but do not provide other benefits. Although digoxin and beta-blockers help strengthen the pumping action of the heart, these drugs do not exhibit renal-protective activity. Device-based interventions only partially relieve HF conditions
and do not provide any means for the heart to recover from damage. Therefore, improved and more innovative treatment options / strategies need to be explored.

2.3.1 Cyclic Guanosine Monophosphate

The second messenger 3, 5 - cyclic guanosine monophosphate (cGMP) is an essential component of signalling pathways that play important roles in cardiovascular disease, including heart failure [19]. Cyclic GMPs are produced through the action of highly specific guanylate cyclases (GCs). GCs are enzymes that convert guanosine 5-triphosphate (GTP) to cGMP. The following three cGMP pathways are of particular importance to cardiovascular systems:

1) Nitric oxide (NO)-sensitive GCs or soluble GCs: In this case, endogenous NO produced by endothelial cells binds to these soluble GCs to produce cGMP

2) Natriuretic peptide receptor A (NPR-A): NPR-A receptors bind to cardiomyocyte-derived atrial and B-type natriuretic peptides to produce cGMP

3) Natriuretic peptide receptor B (NPR-B): These receptors bind to C-type natriuretic peptides, which are secreted by endothelial cells to produce cGMP
Cyclic GMP produced from these three pathways has various actions on a variety of effector molecules, such as 1) cGMP-dependent protein kinase G (PKG), 2) cGMP-regulated phosphodiesterases (PDEs), and 3) cGMP-regulated cation channels [20, 21]. Consequently, these effector molecules lead to physiological changes, such as relaxing the blood vessel walls (vasodilation), increasing water filtration via kidney (diuresis), and increasing sodium filtration (natriuresis).

Importantly, although a common second messenger activates these effector molecules, this does not necessarily mean that the same physiological changes occur following cGMP production. Cyclic GMP produced by different receptors and their distribution will lead to different downstream activation events [22-26]. This differentiated cGMP action is illustrated by the distinct action profiles of the respective endogenous NPs. This topic will be discussed in detail in the following section.
The cGMP signal can be terminated by two mechanisms:

1) Cyclic GMP hydrolysis into GMP through the action of PDEs

2) Cyclic GMP leaches out of the cell into the intercellular matrix. The majority of the leached cGMP is eventually eliminated through urinary excretion

2.3.2 Natriuretic Peptide Pathways

There are four types of natriuretic peptides (NP) in humans, atrial NP (ANP), B-type NP (BNP), C-type NP (CNP) and urodilatin. ANP and BNP are produced in the heart atria and ventricles, respectively, whereas CNP is derived from endothelial cells [27]. Urodilatin is a variant of the ANP precursor. The amino acid sequences of these endogenous peptides are presented in figure 2-7.

Figure 2-7: Amino Acid Sequences of Endogenous Natriuretic Peptides[28]

NPs are similar in their conformational structure and consist of an active site that is located within a ring structure. The ring structure is established by the presence of a disulfide bond between two cysteine amino acids. NPs are very specific with regard
to their binding receptors. Following secretion from either cardiomyocytes or endothelial cells, NPs circulate in the blood circulatory system until reaching the receptor located on the cell membrane. Both ANP and BNP bind specifically to the natriuretic peptide receptor A (NPR-A), whereas CNP binds only to the natriuretic peptide receptor B (NPR-B)[29]. Once NP binds to the complementary active site on the receptor, the cGMP second messenger is produced. As mentioned previously, the elevation of cGMP levels within the cells leads to favourable physiological changes in the body, which are beneficial to both the cardiac and renal system.

Although these peptides are structurally similar, they exert differentiated physiological responses. Studies have demonstrated that the activation of cGMP through ANP and BNP results in vasodilating, anti-fibrotic [30, 31], anti-hypertrophic, and lusitropic properties [32] coupled with renin-aldosterone suppressing response. Other studies have demonstrated that ANP and BNP possess very potent natriuretic and diuretic functions, which are beneficial to HF conditions [30, 32-34].

In contrast, CNP exhibits properties that are distinctly different from ANP and BNP. Unlike ANP and BNP, CNP exhibits more potent anti-proliferative and fibroblast-inhibitory properties that are particularly important in the attenuation of the remodelling process of the heart [35-37]. Studies have shown that a 14-day continuous infusion of the potent cardiac-protecting CNP in acute myocardial infarcted rats assisted in attenuating the heart remodelling process [38]. CNP also produces a less hypotensive state as it exhibits only veno-dilating properties as opposed to the veno-vasodilating properties of ANP and BNP [35, 36, 39]. As shown in figure 2-7, CNP lacks a C-terminus chain, which has been attributed to the lack of
natriuretic function of CNP [40, 41]. As such, although CNP is a good cardiac-protecting agent, it does not exhibit renal-protective properties.

2.3.3 Commercially-approved NP

Currently, two exogenous NPs, Carperitide (synthetic atrial-NP) and Nesiritide (synthetic B-type NP), have been approved and are commercially available. Both Carperitide and Nesiritide are synthesised using the sold-state peptide synthesis method.

**Nesiritide**

Nesiritide is a recombinant variant of human BNP, which was approved in the US in 2001 for the treatment of acute decompensated HF. While endogenous human BNP possesses a half-life of approximately 22 mins, Nesiritide exhibits a slightly shorter half-life of 18 mins. This exogenous peptide functions in a manner similar to human BNP. Nesiritide was reported to exhibit both cardiac-protecting arterial properties and venodilation with diuretic properties. Additionally, Nesiritide helps improve cardiac output by afterload reduction without increasing the heart rate or oxygen consumption. In addition to cardiac protective properties, Nesiritide also claimed to exhibit a renal-protective effect through its natriuretic function. Currently, Nesiritide is administered only to patients with acutely decompensated HF or patients experiencing dyspnoea.

Several major clinical trials, such as VMAC, FUSION-II and NAPA trials, have evaluated the drug in different settings. The VMAC trial evaluated peptide efficacy on acute decompensated HF patients, whereas FUSION-II trial focused on chronic HF patients [42, 43]. The NAPA trial, on the other hand, involved patients undergoing
bypass surgery [43]. In these trials, Nesiritide was reported as effective at decreasing the pulmonary wedge pressure; however, there was no evidence of beneficial renal effects [44-47]. A possible reason for the lack of efficacy could be the short duration of the treatment. This warrants for a chronic peptide delivery system. Chen et al. reported published a feasibility study demonstrating that chronic subcutaneous administration in both animals and humans was more efficacious than bolus administration [48, 49]. Currently, studies are on-going to evaluate the efficacy of the chronic delivery of Nesiritide.

**Carperitide**

Carperitide, a recombinant variant of human ANP, was approved in 1995 for the treatment of HF in Japan [50]. It has been reported that Carperitide is effective at reducing infarct size in acute myocardial infarcted patient, assessed by the investigation of plasma creatine kinase levels and cardiac function *via* left ventricular ejection fraction [51]. This finding was corroborated in a separate study, which demonstrated that treating acute myocardial infarction patients with Carperitide resulted in a decrease in the left ventricular end diastolic volume coupled with improved ejection fraction [52]. However, currently, this peptide is approved for use only in Japan.

### 2.3.4 Designer Natriuretic Peptides

Given the complexity and diversity of HF condition, it is not surprising that current commercially available NPs are not efficacious or ideal for every patient. Indeed, as discussed, Nesiritide is only effective at improving the hypertensive state in HF condition and does not elucidate the potent natriuretic property that was demonstrated for human BNP. Therefore, attempts have been made to design and
develop new NPs with an improved action profile. Different NP variants have been synthesised by altering the peptide sequence to change the receptor affinity and peptide susceptibility to enzymatic degradation.

Driven by the need for a better and more compelling NP drug, researchers at Mayo Clinic have engineered a novel chimeric natriuretic peptide consisting of 22 amino acids (AA) from human CNP and 15-AA C-terminus of the green-mamba-derived Dendroapis natriuretic peptide or DNP.

The novel NP, intuitively named CD-NP, was synthesised *via* solid phase method on a peptide synthesiser, purified by reverse phase high performance liquid chromatography and verified *via* electrospray ionisation mass analysis. The amino acid sequence of CD-NP is shown in figure 2-8.

![Figure 2-8: Schematic Diagram of CD-NP Natriuretic Peptide[16]](image)

DNP was discovered in 1992 and was found to possess very potent natriuretic and diuretic properties [13]. Functionally, DNP exhibits properties that are similar to ANP and BNP. All three peptides bind with NPR-A and NPR-C receptors but not NPR-A
receptor [13, 53], only CNP binds the NPR-A receptor. Of the three peptides that
bind with NPR-A receptor, DNP demonstrates the highest affinity [53, 54]. The three
peptides exhibit potent natriuretic and diuretic properties that are renal protective. In
addition, it has been reported that DNP possesses beneficial cardiac unloading
action, but this is accompanied by significant hypotensive properties [55, 56].

Another key selection factor for the inclusion of DNP in the chimeric NP was its
resistance to degradation by neutral endopeptidase (NEP), which was attributed to
its long C-terminus. Indeed, the DNP 15-AA C-terminus is the longest of the known
NPs; ANP and BNP C-terminus measures 5 AA and 6 AA, respectively, and CNP
does not contain a C-terminus chain. The C-terminus length is thought to provide
resistance to degradation by NEP, hence, increasing the half-life. This phenomenon
is evident in CNP, which possess the shortest in vivo half-life (2 mins) 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 bi-functional peptide exhibits beneficial cardiac-protective functions and a
minimal hypotensive effect coupled with natriuretic and diuretic properties for renal-
protecting attributes [16, 17].

CD-NP, known commercially as Cenderitide, is currently in phase III clinical trials
[11, 12]. In vitro studies have demonstrated that this designer peptide retains a
number of CNP properties, such as activation of cGMP in cardiac fibroblasts and
anti-proliferative actions. Furthermore, the half-life of Cenderitide (approximately 18
mins) is greatly improved compared with CNP (approximately 2 mins) [57].
Therefore, it was anticipated that this peptide would be efficacious for the treatment of cardiorenal disease states, such as HF and AMI.

Indeed, the half-life of ANP, BNP and CNP are short, 2-5 mins, 22 mins and 2 mins, respectively. Given the short half-life \textit{in vivo} and the low secretion levels, these native peptides are typically ineffective at compensating for the progression of HF syndrome. Therefore, there is an interest in developing exogenous natriuretic peptide delivery systems that will maintain elevated plasma peptide levels for longer periods.

\textit{2.3.5 NP Clearance}

Native NPs are subjected to several inactivation mechanisms, such as receptor-mediated endocytosis, enzymatic hydrolysis (by enzyme neutral endopeptidase; NEP) and renal excretion.

A third receptor, natriuretic peptide clearance receptor C (NPR-C) is known to bind to all of the NPs. This receptor acts as a clearance receptor that regulates the amount of circulating NPs by sequestering bound NPs. Other functions of NPR-C have been determined in recent years [58, 59].

\textit{2.4 \textit{In situ} Polymer Precipitation System}

\textit{In situ} forming implant (ISFI) is a new class of controlled drug delivery system that has drawn much attention in recent years. Unlike traditional pre-shaped parenteral depot systems, these biocompatible solvent-based systems do not require any invasive surgical implantation procedure. The formulation contains a solution of
relatively low viscosity, which allows for its administration via an injection needle and makes the ISFI procedure less invasive and painful. The biodegradability of these 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 tissue engineering, three-dimensional cell culturing, cell transportation, and orthopaedic and dental administrations [60-63].

Typically, following the injection of the ISFI subcutaneously or into muscular or other tissues, the liquid systems interact with the physiological environment leading to changes in the properties of the system. These change in properties, which are often mechanical, lead to the formation of a drug depot. Another important advantage of these systems is biodegradability. Biodegradation refers to a deleterious change in the properties of a polymer because of changes in the chemical structure [64]. The breakdown of the polymeric matrix of the depot is useful in two ways. First, the gradual polymeric degradation of the depot allows entrapped drugs to be released gradually over time. By carefully controlling the polymeric degradation rate, the duration of the drug release can be altered. Second, following the complete degradation of the depot, the degradation by-products are removed via natural pathways, through either liver metabolism or kidney filtration, which means that there is no need for a second procedure to remove the implant.

In situ forming implants can be classified into the following three classes according to the mechanism of implant formation:
1) \textit{In situ} cross-linked polymer systems

These polymer systems make use of the \textit{in vitro} or \textit{in vivo} surroundings to initiate crosslinking. Initiation of the crosslinking process can be through inherent physiochemical differences between the system and the surroundings or through an induced change [66-71].

2) \textit{In situ} solidifying organogels

\textit{In situ} solidifying organogel systems typically comprise water insoluble amphiphilic lipids in a heated solvent. Following cooling of the solvent, the solubility of the lipids or gelator starts to decrease. When this happens, the lipids aggregate to form a three-dimensional implant. This implant is generally formed by a network of intermolecular physical interactions, such as Van der Waals interactions and hydrogen bonds.
Notably, organogels include glycerol fatty acid esters, such as glycerol monooleate (GMO), glycerol monopalmitostearate (Precirol®) and glycerolmonolinoleate.

3) *In situ* phase separation systems:

Because they are easy to formulate and to manufacture, systems that rely on this type of *in situ* forming mechanism are the most popular of the three classes. In this system, the gel interacts with the surroundings, either *in vitro* or *in vivo*, to initiate a phase separation process that results in the formation of a solidified depot [72]. ISFIs are suitable for both local and systemic administration of substances with antimicrobial activity, antitumor substances, hormones, substances interacting with the immune system, growth factors, and so forth.

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

2.4.1 *Mechanism*
In situ polymer precipitation drug delivery system is based on the principle of polymer precipitation through water-solvent exchange. This delivery system constitutes a biodegradable polymer in a biocompatible solvent in which drug is dissolved or suspended. The resultant drug + polymer + solvent gel solution is administered by parenteral injection through a syringe needle. Typically, parenteral injections are performed intramuscularly, intradermally or subcutaneously.

Following injection into an aqueous environment, the water-miscible solvent from the gel solution interacts with the aqueous-based physiological surroundings by undergoing solvent-water de-mixing. In this de-mixing process, the water-miscible solvent leaches out of the gel solution into the surroundings, while water from the surrounding tissues migrates gradually inwards. Once a critical water concentration is achieved in the gel system, the hydrophobic polymer begins to precipitate. This polymer precipitation is known as polymer phase inversion, and the critical water concentration is approximately 30% water content [29]. Phase inversion is a critical step in the formation of the polymer matrix. The exact manner in which the injected
gel solution responds to its physiological surroundings determines the final depot morphology and the release characteristics of the gel depot.

2.4.2 Polymer Phase Inversion Dynamics

The manner in which the injected solution interacts with its physiological surroundings affects the dynamics of the phase inversion process. This, in turn, affects the morphological properties of the final gel depot, which will consequently determine the drug release characteristics. As such, an in-depth understanding of the phase inversion dynamics and gel depot morphology will allow the formulation of an effective injectable delivery system.

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

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

2.4.2.1 Liquid-Liquid Phase Separation Rate

Liquid-liquid (L-L) phase separation rate refers to the rate at which solvent from the gel solution effluxes out into the surroundings. This L-L phase separation directly affects the rate of polymer precipitation, resulting in the formation of a variety of shell structures.

**Fast Gelling System**

Systems that contain a hydrophilic solvent with a high water affinity exhibit rapid water absorption and solvent efflux. This forms a fast gelling system characterised
by the formation of relatively hard polymer matrices with interconnecting highly porous macro-voids. The macro-voids in these systems are typically identifiable by their tear-shape or long columnar structures.

Typically, drug release from a fast gelling system is characterised by a large initial burst of drug release followed by a prolonged period of depressed low-level drug release. Polymer-lean voids exhibit a relatively high drug diffusivity ($10^{-5} \, \text{cm}^2/\text{s}$) compared with the lower drug diffusivities ($10^{-7} \, \text{cm}^2/\text{s}$) of polymer-rich matrices [74, 75]. Therefore, during the initial drug burst release phase, the presence of interconnected macro-voids makes it preferable for a drug to diffuse out of the system. The drugs that are not released during the initial burst release phase are usually entrapped in the polymer matrix. The low drug diffusivities of the polymer matrix delay drug diffusion, and the subsequent drug release is expected to be low until the onset of polymeric degradation.

![Diagram](image)

**Figure 2-11: Comparison of Diffusional Path between Fast and Slow Gelling System[76]**

**Slow Gelling System**

In contrast, systems that consist of relatively hydrophobic solvents with a low water affinity demonstrate a slower water absorption and solvent efflux. These conditions form a slow gelling system characterised by the formation of more fluid-like polymer
matrices with small isolated voids. The voids in these systems are typically smaller and are not interconnected.

Drug release from a slow gelling system is typically characterised by a suppressed initial burst release followed by a uniform drug release profile. The presence of smaller and non-interconnecting voids means that the drug release can occur only by diffusion through the polymer-rich matrix (of relatively lower drug diffusivity), which results in the depressed initial burst release. Similar to the fast gelling system, following the initial burst release phase, entrapped drug gradually diffuses through the matrix out of the depot. In this case, the presence of higher levels of entrapped drug leads to a more uniform and consistent drug release. This consistent drug release continues until the onset of matrix degradation after which the drugs remaining in the matrix are rapidly released.

Comparison of fast and slow gelling systems

As discussed, drug release characteristics are highly dependent on the type of gelling system used. In fast gelling systems, the drug release profile is controlled fundamentally by the phase inversion dynamics. This process is governed by the formation of interconnected macro-void structures through which the drug transfer occurs. Little drug transportation occurs through the solidfied, polymer-rich phase. In slow gelling systems, the drug release profile is less critically affected by the inversion dynamics than by the mass transfer kinetics. The notable presence of small isolated pore structures means that the drugs have to diffuse within the continuous, polymer rich phase.
It should also be noted that whereas the initial drug release characteristics are strongly influenced by the system gelation rate, the long-term transport characteristics are a function of the final depot morphology.

### 2.4.2.2 Water Influx Rate into the gel

Water influx rate refers to the migration of water from the physiological surroundings into the depot and the subsequent accumulation of water in the depot. The amount and rate of water influx are crucial because a critical water concentration of 30% is required for the polymer phase inversion to take place. The speed at which this critical water concentration is reached determines the type of phase inversion process and depot morphology, therefore, affecting the type of drug release profile. If hydrophilic drug is of interest, this water influx rate is even more crucial because it determines the rate at which the drug dissolves and therefore how readily it diffuses within and out of the gel implant [76, 77].

The technique for measuring water influx was demonstrated previously by McHugh et al [19, 20]. Briefly, the water influx rate is quantified using a dark ground optics imaging system. In this system, the gel solution is allowed to come into contact with a water reservoir. A video imaging system is set up to capture striations in the dark ground image. These striations represent interference fringe in which the positions are related to the distance the water has penetrated into the solution. From the interpretation of the striations, a concentration gradient caused by water diffusion into the polymer solution is determined.

### 2.4.3 Parameters affecting Phase Inversion Dynamics
Since the inception of *in situ* polymer precipitation delivery system, many different parameters have been investigated, such as solvent and polymer concentration and polymer type, which were found to be relevant to understanding how phase inversion dynamics are affected.

### 2.4.3.1 Effect of solvent

Polymer phase inversion dynamics are critically dependent on the affinity between the solvent and water. Solvents with various water affinities, such as n-methyl-2-pyrrolidone (NMP) [76-79], dimethyl sulfoxide (DMSO) [79, 80], glycofurol [81, 82], triacetin [78, 83, 84], triethyl citrate [85], ethyl benzoate [77, 78, 86], benzyl benzoate (BB) [78, 87, 88], benzyl alcohol (BA) [78], propylene glycol, acetone, tetrahydrofuran (THF), 2-pyrrolidone [89], ethyl acetate [90] and low molecular weight PEG [91] have been investigated extensively.

Hydrophilic solvents such as dimethyl sulfoxide (DMSO), N-methyl-2-pyrrolidinone (NMP) and benzyl alcohol (BA) exhibit high affinity for water. The low miscibility gap between the solvent and water results in a rapid solvent efflux from the gel solution and an increase in water absorption into the gel solution. This produces a fast gelling system, which rapidly forms a semi-solid polymer matrix.

Solvents such as benzyl benzoate, ethyl benzoate, triacetin, triethyl citrate or benzyl alcohols, are more hydrophobic in nature and exhibit a larger miscibility gap with water. Therefore, these solvents tend to leach out of the system at a much slower rate, which in turn retards water ingress, and the resulting polymer matrix tends to be a semi-fluid polymer matrix.
These types of single solvent systems tend to provide drug release characteristics that are often too extreme, exhibiting a release profile that is too slow or too fast. The 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 hydrophilicities at different solvent ratios allows drug release profile to be altered to the desired drug release rate [92-94].

2.4.3.2 Effect of polymer type and concentration

The choice of polymer affects the speed at which gelation takes place. Various types of polymer can be used in these systems. The typical biodegradable polymers used include polylactide (PLA), poly(lactide–co-glycolide) (PLGA), poly(ε-caprolactone) (PCL) [86], polyhydroxyacids, polyanhydrides and polyorthoesters, which can be used as carriers. The PLA, PLGA and PCL polymers are usually preferred because of their clinical precedence [95]. Polymer hydrophilicity plays a critical role in promoting fast water ingress into the system [76, 77, 96].

Generally, the polymer type and concentration affects the initial drug release profile [97-102]. Typically, increasing the polymer concentration in the gel formulation leads to a reduction in the initial drug burst release [76, 97, 100, 103-106]. Conventionally, a 40-50% polymer concentration is preferred. However, the higher the concentration, the more viscous the gel solution becomes, which affects the injectability of the solution; therefore, a compromise must be made.
Studies have investigated the addition of a hydrophilic polymer into the gel formulation. One study demonstrated that the addition of a 3% hydrophilic polymer (polyvinylpyrrolidone, PVP) drastically altered the initial drug burst release. Whereas the addition of PVP did not have an effect on the water influx rate, this polymer has been shown to increase the L-L phase separate rate, which caused an increase in the initial drug burst release [107-110].

2.4.3.3 Effect of Drug

Drug component in the gel formulation can also have a profound effect on the water absorption into the depot. Many different types of drugs have been investigated in in situ forming polymer precipitation systems. These drugs include small molecules, such as lidocaine [111], aspirin [112], peptides [113, 114] and proteins [78, 90]. The effect of the affinity of the drug for the solvent-water plays a critical role in the burst release [76, 77, 115]. Drugs that are hydrophilic in nature have been shown to increase the amount of water influx; however, this 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 phase inversion, water influx and drug release profile.

Effects of gel solution aging and the resultant release characteristics have been investigated [88]. A gel solution that has undergone aging tends to exhibit a network formation. Such structure formation increases with time of aging and is less
pronounced when a ‘good’ solvent is used; however, the formation of the network structure is thermoreversible.

The effects of aqueous content in the gel solution has also been reported to affect the drug release profile [76]. When a small percentage of water is added to the gel formulation, it promotes a faster phase inversion process with a lowering of the water influx. The presence of water in the gel formulation assists in bridging the solvent-non-solvent miscibility gap, which speeds up the phase separation process and brings about the increase in the initial drug burst release.

2.4.4 Advantages and disadvantages of ISPP

2.4.4.1 Advantages

There are several advantages to using in situ polymer precipitation as a controlled drug delivery system. First, its injectability as the mode of administration allows greater ease of depot deployment compared with surgically implanted systems. However, careful consideration has to be taken when formulating the gel solution with respect to gel viscosity. Second, a single gel injection can be formulated to contain a high dosage. By altering the gel formulation, this delivery system minimises the drug burst release and ensures a prolonged yet sustained drug release, which effectively reduces the frequency of repeated dosing for patients on long-term medications. This system not only promotes patient compliance, it also improves quality of life. Third, the manufacturing of the gel is relatively simple, and finally, the creative use of a biocompatible yet biodegradable host polymer means that this delivery depot eliminates the need for a second surgical procedure to remove the implant.
2.4.4.2 Disadvantages

Although the advantages and simplicity of the \textit{in situ} polymer precipitation delivery system have attracted much attention, a number of difficulties are associated with the use of these systems.

The most widely debated problem is the solvent toxicity. This system requires the removal of solvent for the polymer phase inversion to take place. Solvent that enters into the physiological surroundings can potentially cause unwanted local irritation because of the presence of high amounts of organic solvent. This issue becomes even more compelling by the publication of toxicity studies reporting conflicting data [116-119]. This will be discussed further in the next section. Another problem is the reliability of the formation of the implant with respect to size, shape and structure. As discussed, the solvent exchange kinetics and the associated drug release depend strongly on the resulting implant surface area. By varying the size and shape of the implant, this could alter the drug release kinetics [120-122]. For drugs with a narrow therapeutic window, this issue should be considered carefully.

![Figure 2-12: Sources of variability of implant shape and size[65]](image-url)

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<td>Local pH</td>
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Finally, as with most drug delivery systems, it is critical to maintain drug stability. The organic solvents used might denature the more sensitive drugs, which potentially limits the type of drugs and applications suitable for use in this type of delivery system.

2.5 Solvent

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

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 by far the most popular organic solvent because of its pharmaceutical precedence [95]. NMP is a powerful, aprotic solvent with high solvency and low volatility. This colourless liquid has a high boiling point (202°C), high flash point and a mild amine-like odour. NMP is a small cyclic amide with the structural formula C_5H_9NO and exhibits high chemical and thermal stability.
NMP is also known by several other names, such as 1-methyl-2-pyrrolidinone, 1-methyl-5-pyrrolidinone, 1-methylazacyclopentan-2-one, 1-methylpyrrolidinone, 1-methylpyrrolidone, methyl-pyrrolidone, N-methyl-2-pyrrolidinone and 1-methyl-2-pyrrolidone.

NMP demonstrates excellent miscibility with water at all temperatures. It serves as a cosolvent with low molecular–weight alcohols, ketones, glycol ethers and aromatic/chlorinated hydrocarbons. The solvent exhibits excellent miscibility with a variety of other solvents, which makes it a suitable solvent for many different applications. NMP is used extensively in petroleum, electronics, paint, textiles, rubber and chemical polymer fields in industrial applications [123, 124]. Coupled with its excellent pharmaceutical solvency and biodegradable properties, NMP is increasingly used in pharmaceutical applications.

In pharmaceutical applications, NMP is widely used as follows:

1) As a solvent or cosolvent [125-129]
2) As a penetrator enhancer [130-135]

2.5.1.2 Absorption, distribution, metabolism and excretion properties
Chapter 2: Literature Review

Absorption: NMP is an excellent skin penetrator that is easily absorbed into the body via the skin and gastrointestinal and respiratory tracts [136]. Indeed, the permeability rate of NMP is much faster than that of other commercial solvents [137].

Distribution: Following absorption, NMP is distributed in most organs, particularly in the sexual organs. The volume of distribution ($V_d$) of NMP is 0.7 L/kg, and its plasma half-life is between 4 and 12 hours, depending on the mode of exposure [138].

Metabolism: NMP is eliminated from the body through biotransformation into other compounds. As shown in figure 2-14, NMP is readily hydroxylated to 5-hydroxy-$N$-methyl-2-pyrrolidone(5-HNMP), and in turn, 5-HNMP is oxidised to $N$-methylsuccinimide (MSI). MSI is then re-hydroxylated to 2-hydroxy-$N$-methylsuccinimide (2-HMSI)

![Diagram of NMP Metabolic Pathway](image)

*Figure 2-14: Illustration of NMP Metabolic Pathway[139]*
Elimination: The main elimination route for NMP and its metabolites is \textit{via} the kidney through urinary excretion [136]. The elimination of NMP is a saturable process, and unchanged NMP is intensively reabsorbed by the glomeruli[138].

2.5.1.3 NMP Solvent Toxicity

NMP is listed in a number of pharmacopeia. The United States Food and Drug Administration (US FDA) approves a daily exposure of 5.30 mg/day for NMP and classifies NMP as a class-2 solvent. NMP is also approved by the FDA as a constituent of medical devices and is included in the list of approved indirect additives in food-contact materials.

Despite extensive NMP toxicity studies performed on living organisms, including mice[140, 141], rats[140-148], dogs[149], and humans[150-157], there are few studies documenting the toxicity of NMP with respect to subcutaneous or muscular toxicity. Toxicity studies pertaining to subcutaneous or muscular tissue often produce conflicting and contradicting data. For example, it was reported that NMP, DMSO and 2-pyrrolidone induced damage to the muscle tissue when injected intramuscularly into Sprague-Dawley rats. It was concluded that these solvents exhibit myotoxicity [118]. However, another NMP biocompatible study performed in rhesus monkeys reported no acute toxicity. Tissue responses, such as swelling, redness and irritation, were noted to be similar to the response to other biodegradable polymers [116].

2.5.2 Triacetin
2.5.2.1 Physicochemical Properties

![Chemical Structure of Triacetin](image)

Figure 2-15: Chemical Structure of Triacetin

Triacetin is a triglyceride and is the shortest-chain fatty acid ester of glycerol. This solvent is colourless, has a boiling point of 258°C and is an oily liquid with a fatty odour. Triacetin is only slightly miscible with water at approximately 7%, and it readily hydrolyses into acetic acid and glycerol at neutral to alkaline pH values. The half-life at pH 7 and 9 was reported to be 60.4 days and 16.5 hours, respectively, at 25°C. Triacetin is stable at pH 4.

Given its relatively safe toxicity profile, triacetin is used in various industrial applications such as in foods as a flavouring agent and adjuvant, formulation aid, humectant, solvent and vehicle; in cigarette filters and cellulose nitrate as a plasticiser; in the manufacturing of celluloid, photographic films, and fungicides, as a solvent; and as a fixative in perfumes.

In pharmaceutical applications, triacetin is used as a hydrophilic plasticiser in the polymeric coatings of capsules, tablets, beads, and granules, with typical
concentrations of 10% to 35% w/w. Triacetin is also used as an active ingredient in antifungal drugs for the treatment of superficial fungal infections of the skin.

2.5.2.2 Absorption, distribution, metabolism and excretion properties

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

Distribution: The distribution of triacetin following absorption has not been reported; however, triacetin has been reported as a source of liver glycogen.

Metabolism: Similar to most fats, triacetin is readily hydrolysed into glycerol and acetic acid. Triacetin is also hydrolysed by human intestinal lipase; however, it should be noted that triacetin hydrolysis ceases at pH values lower than 4.0.

Elimination: Glycerol is used by the liver and adipose fat tissues as a precursor for metabolic pathways or for storage. The main elimination route for acetic acid is via kidney through urinary excretion.

2.5.2.3 Triacetin Solvent Toxicity

The FDA has listed triacetin as a GRAS (generally regarded as safe) ingredient. The toxicity evaluation of triacetin by the Federation of American Societies for Experimental Biology (FASEB) demonstrated that triacetin was free of toxic effects in long-term feeding and mutagenicity tests. In animal studies, acute oral and dermal
toxicity were very low with no mortality or signs of systemic toxicity. In addition, in human volunteers triacetin did not produce skin irritations or skin sensitisation.

2.6 Commercially Available ISPP System

Currently, three main drug delivery systems use the principle of in situ phase separation, namely, the Atrigel® drug delivery system, Alzamer® depot technology and Saber™ depot technology. Of these systems, only Atrigel® is a commercially available, FDA-approved product.

2.6.1 Atrigel® Drug Delivery System

The Atrigel® technology, developed by ATRIX Laboratories, is based on a biodegradable PLA/PLGA polymer. The delivery system is available as FDA-approved products in pre-filled plastic syringes – Syringe A pre-filled with ATRIGEL® Delivery System & Syringe B pre-filled with leuprolide acetate powder. Contents of both syringes are thoroughly mix by pushing contents of both syringes in a back and fro motion for approximately 45 seconds to obtain a uniform suspension. Resulting suspension will appear colourless to pale yellow and injected parenterally.

The FDA-approved products that utilise Atrigel® technology are as follows:

1) Eligard® - Management of prostate cancer. This is a LHRH agonist leuprolide acetate (1209.4g/mol) controlled release formulation, designed to reduce the amount of testosterone in the body. It is the most successful Atrigel® system and is available in 1-, 3-, 4- and 6-month doses [95, 97, 101, 113, 158].
2) Atridox® - Treatment of chronic periodontitis. This is a subgingival controlled release formulation which contains 8.5% doxycycline (444.43g/mol), which is released over a one-week period [159-161]

3) Atrisorb® - A Guided Tissue Regeneration (GTR) barrier product that does not contain any drug and is used for the guided regeneration of periodontal tissue [162]

4) Atrisorb D – A product containing doxycycline (444.43g/mol) for periodontal tissue regeneration [163]

5) Doxyrobe® - A product containing doxycycline (444.43g/mol) for the treatment of periodontal disease in companion animals

### 2.6.2 Alzamer® Depot Technology

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

### 2.6.3 Saber™ Depot Technology

The Saber™ depot technology system uses a non-polymeric high viscosity carrier, such as sucrose acetate isobutyrate (SAIB), loaded with the intended drug [164, 165]. Developed by DURECT Corporation, the non-polymeric SAIB carrier forms a low viscosity solution when dissolved in organic solvents, such as ethanol, NMP,
triacetin and propylene carbonate. The reported advantage of this technology over the Atrigel® system is the reduction in solvent usage. Typically, in the Eligard® system, 40-50% NMP is used in the formulation, whereas for Saber™ based systems, the solvent use can be reduced to between 15% and 35%. Currently, two products are undergoing clinical testing, POSIDUR™ and Relday™. POSIDUR™, which is currently in phase III clinical trials, is a long-acting local anaesthetic (bupivacaine) developed for the treatment of post-surgical pain. Administered during surgery at the surgical site, POSIDUR™ provides constant local analgesia for up to 72 hours. Relday™, which was developed to treat schizophrenia or schizoaffective disorder, has recently been entered for clinical trial.
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 Aldrich and Fisher Scientific, respectively. Both solvents are of low toxicity and are recommended for use in in situ forming drug delivery systems. CD-NP peptide drug was supplied by Nile Therapeutics, Inc. Pre-mixed buffer solution pH 7.4 was purchased from Ohme Scientific Pte. Ltd.

For the purpose of this work, the preferred delivery system was one that could provide a sustained peptide release for approximately 1 month. Therefore, a rapidly degrading biodegradable polymer was used. Low molecular weight ($M_w$), random copolymer poly (D,L-lactic-co-glycolic acid) 50/50, termed PLGA 50/50, was used as the polymer matrix. The biodegradable and biocompatible PLGA 50/50 was purchased from Purac Biomaterials (Gorinchem Netherlands). The number 50/50 following the description of the copolymer represents the D,L-lactide and glycolide monomer molar ratio. The inherent viscosity of this random copolymer was 0.2 dl/g. To promote faster degradation, the low $M_w$ PLGA 50/50 containing carboxylic acid as the end group was
used. Addition of carboxylic end group will increase polymer hydrophilicity, speeding up the degradation profile.

3.2 Injectable Gel Synthesis

CD-NP was first dissolved in NMP solvent. The solution was allowed to stir for at least 3 – 4 hours before the addition of pre-weighed polymer and triacetin solution. The polymer solution and drug concentration were prepared by weight percentage. The final solution was allowed to homogenise 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 drug release study. On the day of the release, gel solution was drawn into a 1-ml disposable syringe. Precautions were taken to ensure that no bubble was present in the syringe. The gel solution (0.2 cc) was injected into 5 ml of buffer solutions (pH 7.4) through a 20G needle tip. The sample vials were placed on a 3-way rotator set at 150 rpm and incubated at 37°C. On each pre-determined sampling day, the release medium was collected and was replaced with 5 ml of fresh buffer solution. The collected medium samples were tested for peptide concentration using Micro BCA Protein Assay Kit (purchased from Thermo Scientific, Pierce Protein Research Product) [166]. For the release study in which the eluted solvent quantification was required, 1-ml aliquots of the collected released medium were used for solvent quantification using HPLC.
Micro-BCA Protein Assay Kit

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

Briefly, the micro BCA kit consists of 3 reagents, which are mixed at a specific ratio to formulate a working reagent. To measure the protein concentration, equal amounts of the sample and working reagents were mixed and were incubated for 1 hour at 60°C. The samples were allowed to cool to room temperature before running through the spectrophotometer at 562 nm absorbance. A blank sample was run before the first of the experimental samples to establish the baseline absorbance at 562 nm. To improve the accuracy of the measurements, a set of 7 peptide standards was measured at every release time point for equipment calibration.

3.3.2 Solvent Efflux Study

Solvent efflux quantification was determined using HPLC. The procedures for the solvent efflux study were based on the release study protocol discussed in section
3.3.1. At pre-determined time points, samples of the released medium were collected into glass sample vials, and the medium was replaced with fresh buffer solution. The medium samples were filtered through a 0.22-um syringe filter into HPLC sample glass vials. The prepared samples were transferred onto a HPLC sample tray and were maintained at 4°C.

The same HPLC system used for peptide stability study was used for solvent quantification. The operating conditions of the HPLC were as follows: 30-ul sample injection volume and a mobile phase consisting of 40% ACN and 60% H2O, with a flow rate of 1.0 ml/min at a detection wavelength of 210 nm. The run-time was set at 6 mins; NMP and triacetin were expected to be eluted at 3.6 mins and 4.6 mins, respectively. Detailed HPLC working conditions are provided in Section 4.2.

3.3.3 Morphological Study

Both scanning electron microscopy and optical microscopy were used to evaluate morphological analysis and shell structure porosity. Shell porosity is directly related to the rate of gelation. As such, analysis of shell porosity in various gel formulations will enable better understanding of gel formulation and its associated drug release profile. Shell structure formation analysis will also be critical at understanding release mechanism from such system.

The gel solution was injected and allowed 3 days of immersion in the buffer solution for the outer shell structure to form and thicken. The semi-solid implant was removed and
was rinsed twice with deionised water to remove any salt residue on the implant surface. Following rinsing, the implant was frozen immediately in liquid nitrogen for 1 min and fractured to expose the cross-sectional area. The sectioned samples were lyophilised for 7 days using a laboratory freezer dryer (Telstar Industrial, Spain) before the shell-structure analysis was performed using scanning electron microscopy (JEOL Ltd., Japan).

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

3.3.4 Contact Angle Measurement

Hydrophilicity of the gel formulation is directly related to the contact angle. The higher the hydrophilicity is, the lower the contact angle. Contact angle was measured using First Ten Angstrom, FTA32. Gel formulation was drawn into a syringe and attached onto the equipment. The syringe plunger was precisely depressed using a computer-aided mechanical pusher. The pusher was stopped when a drop of gel formulation detached from the needle tip and land on a glass slide. Immediately after the gel formulation hits the glass slide, the contact angle was measured using FTA32 software. The process was repeated 10 times and the average contact angle was taken to be the contact angle.
for that gel formulation. A detailed explanation and the results will be discussed in detail in Section 5.4

3.3.5 Water Uptake Study

The procedures for the water uptake study were performed based on the release study protocol discussed in section 3.3.1. At pre-determined time points, the implant was removed from the buffer solution and was rinsed with deionised water to remove any salt residue on the implant surface. Following the washing process, the water residue on the implant surface was blotted off using laboratory Kimwipes (Kimberly-Clark, USA), and the implant weight was recorded. The water uptake percentage was calculated based on the following equation:

\[
\text{Water Uptake} \% = \frac{W_{\text{wet}} - W_{\text{polymer}} - W_{\text{NMP}} - W_{\text{triacetin}}}{W_{\text{polymer}} + W_{\text{NMP}} + W_{\text{triacetin}}} \times 100\%
\]

Where

- \( W_{\text{wet}} \) = wet weight of gel at respective time points,
- \( W_{\text{polymer}} \) = weight of polymer,
- \( W_{\text{NMP}} \) = weight of remaining NMP,
- \( W_{\text{triacetin}} \) = weight of remaining triacetin

The equation considers the weight fluctuations as a result of the water uptake and solvent efflux. At each time point, water ingress, remaining NMP and triacetin in the gel implant together with polymer weight contributed to the measured wet weight. The polymer weight was assumed to be constant throughout the first week until the onset of degradation. The buffer medium was tested for NMP and triacetin as per the solvent
efflux protocol. The solvent efflux quantification allowed for the indirect derivation of the remaining solvent.

3.3.6 Degradation Study

The degradation study was performed in accordance with the release study protocol described in section 3.3.1. At each pre-determined time point, 3 gel samples were removed from the sample batch. The removed samples were lyophilised for 7 days using a freeze dryer before the samples were prepared for polymer molecular weight analysis.

The lyophilised samples were dissolved in chloroform to produce the desired polymer concentration of approximately 5 mg/ml. The dried samples were maintained in chloroform for 24 hours to ensure the complete dissolution of the polymer. Following the 24-hour dissolving process, the resultant PLGA/chloroform solution was filtered through a 0.22-µm syringe filter into an SEC sample vial.

The polymer molecular weight analysis was performed using size exclusion chromatography (SEC) on a 1100 series SEC system (Agilent Technologies). The system consisted of an 1100 series solvent delivery system, sample injector, refractive index detector and a 300 mm x 7.8 mm PLgel mixed-C, 5-µm column. The operating conditions for the SEC setup were as follows: 50-µl sample injection volume and a mobile phase consisting of 100% chloroform with a flow rate of 1.0 ml/min. The
molecular weight calibration was established using a series of polystyrene standards 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 rat studies were performed at CardioRenal Research Laboratory, Mayo Clinic (Rochester, MN). The Mayo Clinic Animal Care and Use Committee approved the in vivo rat study protocols (Mayo IACUC number: A34809).

Male Wistar rats (Charles River Laboratories, Wilmington, MA), weighing 150 – 250 g, were used for the rat studies. The rats were maintained on a standard laboratory diet and were allowed at least 3 – 4 days to acclimatise to the animal facility housing prior to the start of each study. Throughout the entire study, the rats were placed in pairs in specialised rat housing to ensure sufficient roaming space. The rats were kept in rooms timed with a 12-hour light cycle to promote a normal rodent activity cycle.

3.4.2 Blood Pressure Measurement

The rodent blood pressures were measured using the CODA non-invasive blood pressure system for mice and rats (Kent Scientific Corporation). The blood pressure system comprised a series of 6 cylindrical tubes in which the rodents were restrained. Tail cuffs were applied from the end of the tail up to the thickest portion of the tail. The rats were acclimated to the internal environment of the cylindrical containers. To help
the rodents calm down, the blood pressure measurements were taken in the dark or under minimal lighting. Once all 6 rats were calm enough, the blood pressure software 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 and the systolic, diastolic and mean blood pressure. The rats that became agitated during the measurement process were removed, placed back in the rat housing, and allowed to calm down before the measurements were retaken. The entire process was repeated until all of the rats’ blood pressures were measured successfully. Once this was achieved, the rodents and their housings were returned to the rat housing facility.

3.4.3 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 through the carotid artery, and the animals were sacrificed after the blood was collected. Before the start of the collection procedure, the rodents were laid belly side up on a rat operating table and anaesthetised using isoflurane (1.5% in oxygen). The hairs on the neck were shaved to allow easy access to the underlying tissues. A small incision was made in the neck, exposing the subcutaneous fat layer. The fat layer, followed by the muscle layer, was teased away carefully to expose the carotid artery. Restraint clips were placed up- and down-stream of the intended point of incision. Following the incision, PE-50 tubing was inserted into the carotid artery and was tied down using a suture. A 3-ml syringe was attached to the open end of the PE-50 tubing to commence the blood collection. This process was repeated until all the blood was collected, and the rodent was deemed
dead once it stopped breathing. The collected blood was placed immediately in EDTA tubes on ice. The tubes were centrifuged at 2,500 rpm at 4°C for 10 minutes to separate the plasma and other blood constituents. The plasma was aliquoted and stored at -80°C until peptide or cGMP testing.

Radio Immunoassay Kit C-NP and cGMP

Radio-immuno assay (RIA) kits (Perkin Elmer Life Science, USA) were used to evaluate the CD-NP and cGMP concentration [167]. The C-NP and cGMP RIA kit was used for CD-NP and cGMP quantification, respectively.

The CNP RIA assay is based on binding competition between the radioactive and non-radioactive peptide found in the sample. In this case, the competition was between $^{125}$I-CNP and CD-NP. The limited quantity of antibodies specific for the peptide restricted the amount of binding sites. As the quantity of the peptide standards or unknown samples increased, the amount of $^{125}$I-CNP peptide able to bind with the antibody decreased. Quantification of the amount of $^{125}$I-CNP as a function of the peptide concentration was established using a gamma counter machine. From this calibration function, the peptide concentration in both the plasma and urine was calculated. This assay required two overnight incubations at 4°C. The cGMP RIA assay works on similar principle except that the peptide is replaced with appropriate antigens.

3.4.4 Urinary Output Evaluation
Urinary output was analysed for CD-NP 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 chilled beaker. Evaporation of the collected urine was minimised by maintaining the chilled beaker 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 aliquoted for peptide and hormone analysis. The same RIA assays used to measure the plasma CD-NP and cGMP were used to measure the CD-NP and cGMP levels in the urine.

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

### 3.4.5 Statistics Analysis

All statistical analysis was performed using GraphPad Prism 5.0. All *in vitro* data are expressed as mean ± SD, whereas the *in vivo* data are expressed as mean ± SEM. Statistical comparison between groups was performed by t-test. P-value < 0.05 was accepted as significant difference.
4.1 CD-NP Analysis

4.1.1 CD-NP Quantification

3 protein assay kits were selected to quantify CD-NP peptide concentrations. These kits include Micro BCA Protein Assay Kit, BCA Protein Assay Kit and Modified Lowry Protein Assay Kit. Several trials were conducted using these kits to test for its suitability at detecting CD-NP. Ultimately, Micro BCA Protein Assay Kit was identified to provide the highest sensitivity and accuracy at detecting the short peptide. A detailed explanation on the workings of this assay kit was described in section 3.3.1. This method of quantification was used in all peptide release studies conducted in this work.

To further minimize any measurement errors, a set of 7 standards was tested for every peptide release timepoint. Peptide standards ranged from 0.5ug/ml to 20ug/ml. Figure 4-1 shows the calibration plot for one of the calibration run.
4.2 Solvent Analysis

4.2.1 Solvent Stability

A suitable method for solvent quantification has to be identified before the commencement of solvent stability calibration and solvent efflux from the gel systems.

High Performance Liquid Chromatography (HPLC) was identified as an appropriate testing method and work was carried out to develop a suitable HPLC method that could distinctly resolve both the solvents employed in the co-solvent systems for quantification purpose [168]. Below shows the final optimized HPLC method used for the testing of NMP and triacetin.

HPLC Column Manufacturer: Agilent

HPLC Column: Zorbax 300SB-C-18 Column, 5um, 4.6 x 250mm

Mobile Phase: 40% ACN :60% H2O
Run Time (Stop Time): 6mins
Detection wavelength (nm): 210nm

Figure 4-2 shows the peak definition of both NMP and triacetin. Retention time for NMP and triacetin was identified to be at approximately 3.6mins and 4.6mins respectively. The peak at 3.1mins was contributed by the buffer solution used to dissolve both solvents.

With the final optimized HPLC method, solvent stability was investigated. NMP is known for its excellent thermal and chemical stability as a solvent or co-solvent system. Triacetin, on the other hand, is known to have a short half-live of 7days in pH7.0 water.
Chapter 4: *In vitro* Studies: Drug / Solvent Analysis

To investigate on solvent stability in buffer solution, known [NMP] and [triacetin] of 50ug/ml, 150ug/ml and 250ug/ml was prepared. Samples were incubated at 37°C and rotated at 150rpm. The conditions were as such to mimic the release and degradation study conditions. For 6 consecutive days, 1ml of prepared solutions was extracted and tested using HPLC for solvent concentration quantification. Samples were run in triplicates.

**NMP Stability in Buffer**

Figure 4-3 shows NMP chemical stability in buffer over a 6-days period. As expected, no changes in NMP concentration were observed throughout the entire test duration for all 3 NMP concentrations. This demonstrated that NMP is indeed very stable in buffer.

![NMP Stability Chart](image)

**Triacetin Stability in Buffer**
Figure 4-4 shows triacetin chemical stability in buffer over a 6-days period. Unlike NMP standards, triacetin concentration for all 3 known concentration standards experienced a drop over the entire test duration.

![Triacetin Stability Graph](image)

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

Indeed, it has been reported that triacetin has a short half-life of approximately 7 days in H₂O at pH 7.0. Triacetin hydrolyzes easily into glycerol and acetic acid. From the changes in [triacetin] over the 6-days period, triacetin half-life could be calculated based on the half-life equation shown below:

\[ N(t) = N_0 \left( \frac{1}{2} \right)^{t/t_{1/2}} \]

Table 4-1 shows the calculated experimental triacetin half-lives for all 3 concentrations. At the given conditions (37°C incubation, rotated at 150rpm), triacetin half-life in buffer averaged at 8.78 ± 1.01 days
Chapter 4: *In vitro* Studies: Drug / Solvent Analysis

4.2.2 Solvent Calibration

Following stability study, solvent calibration was established based on the HPLC method described in section 4.2.1.

**NMP Calibration**

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

\[
\text{Injected Gel Volume: } 0.2\text{cc}
\]
Total NMP loading: 80,000ug
Estimated 50% initial burst: 40,000ug
Buffer Volume: 5ml
Estimated highest [NMP]: 8,000ug/ml

A total of 18 standards ranging from 0.5ug/ml to 10,000ug/ml were prepared for the calibration run. 10,000ug/ml standard concentration should exceed the estimated burst concentration while lowest detection limit for NMP via this HPLC method was found experimentally to be 0.5ug/ml. Calibration plot was based on triplicates.

Figure 4-5 shows the NMP calibration curve. It could be observed that absorbance do not correlate linearly with NMP concentration. The absorbance and [NMP] follows a linear correlation between 0ug/ml – 250ug/ml and between 3,000ug/ml – 10,000ug/ml.
In order to preserve a linear correlation, NMP calibration was shorted to 0ug/ml to 250ug/ml. Samples with expected NMP of higher than 250ug/ml will be diluted to within the calibration range. Figure 4-6 shows the corrected NMP calibration.

![NMP HPLC Calibration](image)

**NMP**

HPLC Calibration
0.5ug/ml - 250ug/ml

\[ y = 59.065x + 85.51 \]

\[ R^2 = 0.9993 \]

**Figure 4-6: NMP Calibration from 0.5ug/ml - 250ug/ml**

**Triacetin Calibration**

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

- Injected Gel Volume: 0.2cc
- Total triacetin loading: 40,000ug
- Estimated 50% initial burst: 20,000ug
- Buffer Volume: 5ml
- Estimated highest [triacetin]: 4,000ug/ml
A total of 15 standards ranging from 5ug/ml to 10,000ug/ml were prepared for the calibration run. The lowest detection limit for triacetin is 5ug/ml.

Figure 4-7 shows the triacetin calibration curve. Unlike NMP, HPLC absorbance and triacetin concentration follows a very linear correlation. The HPLC method, however, has a lower detection limit of only 5ug/ml.

4.2.3 Effects of Co-solvent

Following the quantification of the stability and calibration of the respective solvents, the effects of co-solvents were investigated. Two main aspects were looked into: 1) co-solvent homogeneity and co-solvent influence on each solvent.

4.2.3.1 Co-solvent homogeneity
Solvent homogeneity is critical in a co-solvent system. In an ideal system, there should be complete homogeneity between the two solvents employed in the system. This will allow drug and polymer to dissolve homogeneously within the entire gel system. This, in turn, will precipitate to form a polymer matrix where drugs are evenly distributed throughout the entire gel depot, thus allowing the drug release to be not only uniform and predictable and also to achieve consistency between samples.

To test for solvent homogeneity, two co-solvent solutions, 40% NMP / 20% TRI and 50% NMP / 10% TRI, were prepared in buffer. Immediately after mixing via 30 seconds vortex, two samples were carefully prepared for each of the solutions. 1 sample was collected from the solution nearer to the top half of the solution while the other was collected from the bottom half of the same solution. Extracted samples were prepared via HPLC sample preparation protocol and tested on HPLC. The remaining co-solvent solutions were left undisturbed at room temperature for 1 hour. After 1 hour, two samples from the same collection locations were extracted and tested on HPLC.
Chapter 4: *In vitro* Studies: Drug / Solvent Analysis

**NMP Homogeneity in co-solvent system**

<table>
<thead>
<tr>
<th></th>
<th>Top of bottle</th>
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<tbody>
<tr>
<td>40% NMP 20% Tri</td>
<td>15000</td>
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<tr>
<td>50% NMP 10% Tri</td>
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HPLC Absorbance / mAU

**Triacetin Homogeneity in co-solvent system**

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<tr>
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<tbody>
<tr>
<td>40% NMP 20% Tri</td>
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<tr>
<td>50% NMP 10% Tri</td>
<td>200</td>
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HPLC Absorbance / mAU

Figure 4-8: NMP and Triacetin Homogeneity in Co-solvent System
Figure 4-8 shows NMP and triacetin results collected from this study respectively. In both co-solvent solutions, the amount of measured NMP and triacetin were similar between samples analyzed at 0h and 1h. No significant differences were also observed between samples collected from top half and bottom half of the solution. These two observations strongly suggest that the two solvents have good miscibility and that no solvent partitioning was observed at the prepared solvent ratio.

4.2.3.2 Co-solvent Influence

After establishing that there is good homogeneity within the co-solvent solution, another study was carried out to investigate on the influence of one solvent on the other in a co-solvent system.

In this study, two co-solvent solutions were prepared such that there resultant solutions contain 50ug/ml NMP and triacetin or 100ug/ml NMP and triacetin. As controls, pure NMP and triacetin of 50ug/ml and 100ug/ml were prepared. Samples were allowed to rotate at 150rpm at 37°C. At predetermined timepoint i.e. 0d / 1d / 2d / 3d, 1 ml was extracted from each samples and test on HPLC for solvent quantification.
Figure 4-9: NMP and Triacetin influence on each other in Co-solvent Solution

Figure 4-9 shows the results of solvent quantification in this study. The white columns represent the pure solvent samples while the purple and blue columns represent NMP and triacetin in the co-solvent solution respectively.

**NMP Quantification**
As discussed in section 4.2.1, NMP exhibits excellent thermal and chemical stability. As expected, the amount of NMP detected in the pure NMP samples by HPLC was consistent throughout the 3 days. Interestingly, the amount of NMP measured in the co-solvent appeared to be slightly lower than the 50ug/ml and 100ug/ml controls. It appeared that there was some ‘shielding’ effect from triacetin which resulted in the reduced absorbance. This ‘shielding’ effect did not appear to be concentration dependent. For the 50ug/ml co-solvent sample, between 1d to 3d, NMP detection varies by 5.36% to 10.02% as compared to the control the variations for the 100ug/ml co-solvent samples ranged from 5.07% to 7.15%.

However, it is worth noting that this reduction in NMP detection in the presence of triacetin is consistently observed in all known samples that were tested and that the difference between actual and expected concentration typically falls between 5-10%.

**Triacetin Quantification**

The trend observed with the pure triacetin samples is consistent with results discussed in section 4.2.1. Triacetin half-life was found to be approximately 8.7 days in the current experimental conditions. Unlike NMP which is influenced by the presence of triacetin, measured triacetin was not affected by the presence of NMP. In all but 1 instance, triacetin concentrations in the co-solvent solution were correctly quantified by HPLC. This suggested 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 the delivery of CD-NP peptide to treat heart failure in patients, the ideal release profile is one that follows a zero-order release profile over a course of 4 weeks, with little or no initial burst.

A huge initial peptide burst release is undesirable. As discussed, CD-NP peptide exhibits vasodilating properties. A sudden increase in plasma peptide concentration could lead to drastic hypotension in patients, causing discomfort, dizziness and, in the worst scenarios, white-out or even death. Therefore, it was critical to avoid this situation by maintaining the initial burst release at a minimum. Ideally, the initial peptide burst 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, a sudden burst of peptide at any point during the release is undesirable. Again, the effects of an over dose will cause unnecessary discomfort to patients. Second, CD-NP demonstrated a
relatively short *in vivo* half-life of 18 mins. A rapid drug releasing profile would certainly result in unnecessary drug wastage. Peptide that does not bind to NPR-A or NPR-B receptor will likely be deactivated by NPR-C clearance receptors and/or will be cleared by the kidneys.

Many factors contribute to the release profile of a gel system. In this study, the effects of several different gel parameters, namely, the effect of (1) polymer concentration, (2) co-solvent, (3) drug loading and (4) injected volume of the gel were investigated. During the selection of the different parameters, the gel viscosity of the formulation was considered. If a solution is too viscous, it will be difficult to syringe, making it unsuitable as an *in situ* forming application.

All constituent in gel formulations stated in the following sections are expressed as w/w % unless otherwise stated.
5.1.1 Effects of [Polymer]

![Effect of Polymer Concentration Graph](image)

**Figure 5-1: In vitro study: Effect of Polymer Concentration**

Polymer concentration will affect the initial burst effect of the gel system. In this study, gel formulations loaded with 0.15% peptide in varying amounts of polymer and NMP concentrations were synthesized to investigate the effects of polymer concentration on the release profile. Figure 5-1 shows that the initial burst decreased with increasing polymer concentrations. There are two possible explanations for this observation.

First, the presence of a higher polymer concentration in the formulation could have slowed down the phase separation process or gelation rate [76]. This is usually coupled with a slower water influx into the system, producing a relatively slower gelling system, which forms a thicker and denser shell structure that impedes drug diffusivity out of the
gel system. As such, a reduction in the initial burst release is observed. This phenomenon has been reported in several membrane forming systems [103, 104, 109].

Second, the initial peptide release could be ascribed to the solvent efflux from the gel system. The dissolved CD-NP could have eluted out of the system with the rapid efflux of the hydrophilic NMP solvent. In a gel formulation with a higher polymer concentration, the amount of NMP solvent in the system is lower. This means that the solvent efflux out of the system is reduced. Therefore, the amount of dissolved CD-NP eluting out of the system is lowered. Conversely, a formulation with a lower polymer concentration will contain a higher weight ratio of NMP. High levels of dissolved CD-NP could elute out of the system together with the rapid NMP efflux, resulting in a higher initial peptide burst release.

In the subsequent peptide release, the release rate for the system with higher polymer concentration was much faster. In the 30% polymer concentration system, close to 85% of the total drug was released within the first 3 days, whereas only 15 - 18% was released in the higher polymer concentration formulation. As such, the absolute amount of drug remaining in the depot for the former formulation was much lower than for the latter formulations. The lower amount of residual drug at 3 days resulted in a lower drug concentration diffusional gradient, which explains the slower subsequent release rate. Conversely, for the latter system, the presence of a higher residual drug concentration produced a higher drug diffusional gradient and facilitated the diffusional-based release.
5.1.2 Effects of Co-solvent System

As discussed in section 2.4.3.1, the overall solvent hydrophilicity plays a critical role in determining the system gelation rate. In this study, the effect of co-solvent systems consisting of hydrophilic solvent (NMP) and hydrophobic solvent (triacetin) was investigated. This study was performed in two parts; the effect of triacetin at various concentrations and polymer concentrations in a co-solvent system was investigated.

5.1.2.1 Effects of [Co-Solvent]

![Figure 5-2: In vitro study: Effect of Triacetin Concentration](image)

Figure 5-2 shows the release profiles of 0.15% peptide loading in 30% PLGA 50/50 in varying NMP and triacetin concentrations. As expected, the gel formulation consisting of NMP as the only solvent exhibited the highest initial burst release. NMP is a hydrophilic solvent that rapidly demixes with water, leading to high efflux out of the system. This
rapid efflux promotes a fast phase inversion process and high water influx rate. The resultant shell structure is one containing large tear-shaped pores, which are interconnected. This interconnected network of pores allows peptides to diffuse easily out of the system, leading to the high initial burst release.

Following the addition of 20% and 30% triacetin to the formulation, the initial burst release was drastically reduced to 31 ± 4% and 13 ± 4%, respectively. With the addition of triacetin, the overall hydrophobicity of the system was increased. The increase in system hydrophobicity induced a slower phase inversion and water influx rate. This resulted in a shell morphological transition from a high porosity shell structure to one that was denser and less porous. As explained, a slower gelling system tends to consist of small isolated pores within the shell structure. Consequently, the peptide has to travel through the polymer-rich matrix, which exhibits inherently lower drug diffusivity. The slower drug migration rate leads to a reduction in burst release.

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

5.1.2.2 Effects of [Polymer]
Chapter 5: *In vitro* Studies: Injectable Gel System Analysis

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

Figure 5-3 shows the drug release profiles at various polymer concentrations in the co-solvent system loaded with 20% (A) and 30% (B) triacetin.

Previous release studies have demonstrated that both polymer concentration and the addition of triacetin affect the initial peptide burst release. However, despite the variation in polymer concentrations, the co-solvent systems containing 20% triacetin demonstrated an average initial burst of 31 ± 4%, whereas the system containing 30% triacetin demonstrated an average initial burst of 13 ± 4%. This result strongly suggests that shell morphological structures are determined pre-dominantly by solvent hydrophilicity. Particularly in a co-solvent system, the composition of various hydrophilicity solvents will change the overall gel hydrophilicity. This will affect the formation of different shell morphological structures. The effect of polymer concentration in the formulation takes precedence only in fast gelling systems. In other words, the
effects of a co-solvent on the dynamics of phase inversion are more significant than the effects of polymer concentration.

5.1.2.3 Effects of [Drug loading]

Figure 5-4: *In vitro* study: Effect of Drug Loading

20% Triacetin co-solvent system A1) % Cumulative Drug Release A2) Weight of Drug Released

30% Triacetin co-solvent system B1) % Cumulative Drug Release B2) Weight of Drug Released

Figure 5-4 shows the release profiles of 2 co-solvent systems with varying drug loading concentrations. The plots demonstrated that the drug loading did not affect the initial burst release. This was expected because the changes in the drug mass percentage in the formulation relative to that of the polymer were small. These data are consistent with
another study in which a drug loading concentration ranging from 0.15% to 0.45% did not demonstrate an effect on the release profile [169]. However, it has been reported that when the drug mass percentage is large enough, the structure formation during the phase inversion process is affected, resulting in a higher initial burst release [115].

5.1.3 Effect of Injected Volume

![Graph A: Effect of Injected Volume](image)

![Graph B: Effect of Injected Volume](image)

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 a co-solvent formulation containing varying injected gel volumes. The injected volumes investigated in this study were 0.1, 0.25 and 0.5 cc. The initial peptide burst release was similar for all 3 injected volumes. The shape and size of the implant also influenced the drug release profile. As with all membrane-based system, a larger gel surface area in contact with the surrounding area allows the solvent to efflux out of the system faster and more water to influx into the system. In this case, the effect of the differences in the gel surface area did not have an obvious effect on the initial burst release, possibly because of the use of the co-solvent system. Similar to the effects of polymer concentration, the effect of injected volume plays a more
influential role in a single solvent, fast gelling system; the co-solvent formulation dominated the phase inversion process, masking its influence.

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

5.2 Solvent Efflux Study

There are two keys fundamental parameters that will directly affect polymer phase inversion kinetics. These are namely liquid-liquid phase separation rate and water influx rate into the gel. The in vitro results presented in the previous section exemplify the importance of solvent in the phase inversion process. The solvent efflux rate determined the phase inversion process and influenced the water influx rate. Understanding this efflux process helped control the shell structure and therefore the initial drug release. In addition, the in vitro release studies illustrated that the addition of triacetin altered the gel solution hydrophilicity and affected the release profile.
This section investigates and elucidates possible explanations for the changes in the drug release profile with respect to the gel solvent content. There are 2 postulates discussed in this section.

Postulate 1: Initial peptide burst release is directly related to efflux of NMP

Postulate 2: Initial peptide burst release is directly related to the changes in phase separation kinetics

5.2.1 NMP Efflux vs. Peptide Release

Postulate 1:

Initial peptide burst release is directly related to efflux of NMP

This work demonstrated that phase inversion and water influx rate play a critical role in influencing the peptide release profile. However, in all the release studies conducted, a trend was observed suggesting that the increased initial peptide burst release was associated with the presence of increased NMP in the gel formulation. Since CD-NP has a higher solubility in NMP, an increased in NMP content in gel formulation which leads to a higher NMP efflux might result in the observed higher peptide burst release. In this section, whether peptide release is a direct consequence of the NMP content in the formulation was investigated. In total, 5 gel formulations were prepared containing NMP concentrations ranging from 20% to 60%. In the formulations, CD-NP and polymer
loading were maintained at 0.45% and 40%, respectively. Appropriate amounts of triacetin were added to the formulation to make up 100% solvent.

Because only the initial peptide burst release was of interest, data were collected only on the first 3 days. At the respective time points, all of the buffer solution was extracted from the sample bottle and was replaced with fresh buffer solution. The extracted buffer
solution was aliquoted into samples and tested for solvent content using HPLC and for drug content using micro BCA protein assay kit. Figure 5-6 A and B shows the initial 3-day drug and NMP release plots. In figure 5-6 C, the peptide released was compared with NMP solvent efflux amount.

Similar to the *in vitro* release studies, the peptide burst release at day 1 followed an upward trend with increasing NMP concentrations in the gel formulation. The plot of released peptide vs. NMP efflux on day 1 (figure 5-6 C) demonstrated that there was no clear trend suggesting CD-NP is eluting together with NMP efflux. A correlation analysis showed that there is no significant correlation between eluted CD-NP and NMP efflux. Although data did not demonstrate conclusively that little or no dissolved CD-NP was eluted with NMP efflux, the data corroborated that the peptide burst release was not dominated by the dissolved peptide in the NMP efflux. Therefore, this study disproves postulate 1.

In addition, from figure 5-6 C, it can be observed that although 3 gel formulations had NMP released between 51,000 – 56,000ug, CD-NP corresponding burst release was much higher for single solvent system (9,500ug) as compared to the other two co-solvent systems (7,300ug and 5,700ug). This confirms that co-solvent system does have an effect on initial burst release. This will be discussed in the second postulate.

### 5.2.2 NMP vs. Triacetin Solvent Affinity
Postulate 2 states that initial peptide release is related to the changes in phase separation kinetics. This change phase separation kinetics in a co-solvent system could be a result of two possible causes.

Sub-postulate 2A:
Change in phase separation kinetics is a direct result of co-solvent interaction

To understand further the solvent efflux mechanism, a study was designed to investigate whether there was solvent affinity between the co-solvents. The postulate was that if NMP has a higher affinity for triacetin than for water, the presence of triacetin in the co-solvent system would retard NMP efflux, slowing down the phase separation kinetics. This in turns slows down the gelation process resulting in a less porous shell structure. In this study, 2 groups of gel solutions were prepared, 1 group contained 20% triacetin, whereas the other group contained 30% triacetin. Within each group, 3 sub-formulations containing varying amounts of NMP were prepared. In this case, polymer concentrations were added into the formulation to make up to 100% w/w.
Figure 5-7: 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 5-7 A and B shows the daily NMP efflux. The trends observed were expected. Higher amount of NMP loading in the gel formulation were accompanied by higher NMP efflux on day 1. A plot of NMP efflux against NMP loading for both groups, shown in Figure 5-7 C, demonstrated that there was a distinct significant correlation between the two. The red plot represents the linear regression for both sets of data. This study illustrated that NMP efflux was solely dependent on the loading regardless of the amount of triacetin, demonstrating that the presence of triacetin did not contribute to the changes in the NMP efflux amount. Hence disproving postulate 2A.
5.2.3 Overall Gel Hydrophilicity

Previous sections have demonstrated that efflux of NMP from the gel system did not directly affect peptide release. It also disproved postulation 2A that change in gelation rate of a co-solvent system is not a result of retardation of NMP efflux in the influence of triacetin. With these conclusions established, there is another possible reason for the change in phase separation kinetics. Solvent ratios in a co-solvent system will alter the overall gel hydrophilicity. This change in gel hydrophilicity could have an influence on solvent efflux and water influx, leading to changes in shell structure characteristics.

Postulate 2B:

Change in phase separation kinetics is a result of changes in gel overall hydrophilicity in co-solvent systems

Figure 5-8 illustrates two possible scenarios on the effect of co-solvent concentrations in a gel system.
In the first scenario, if both formulations contain the same amount of triacetin but different NMP and polymer concentrations, the formulation with the higher NMP loading should demonstrate a higher NMP efflux. This higher NMP efflux should lead to a more porous shell structure formation. If this is true, the peptide release should also be higher. However, this scenario contradicts the drug release profile, which suggested that the amount of drug released was similar. Therefore, this scenario is unlikely to hold.
In the second scenario, a faster efflux forms a thicker outer shell. As illustrated in the figure, the formulation with the higher NMP loading should form a structure that is more porous. However, given the thickness of the shell, the overall structure impedes or retards the rate of drug release. Therefore, both the higher and lower NMP loading formulation will exhibit a similar initial peptide burst release. In a reported study, the addition of 3% hydrophilic polymer (PVP) into the gel formulation led to an increased in the overall gel hydrophilicity. This resulted in an 8-fold increase in L-L phase separation rate although no changed in water influx rate was observed[76]. This increased phase separation kinetics could have contributed to a thicker shell structure, as described in the second scenario.

To confirm this second scenario, morphological study and gel hydrophilicity study 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 injection of gel solution into the buffer solution. As time progresses, there is a solvent-water exchange between the gel depot and surrounding medium. This influx of water precipitates the polymer to form the outer shell. Whereas the shell assumes a certain degree of structural integrity, the inner core of the gel still contains a moderate amount of gel solution. To mitigate the issue of a harder outer shell and a softer inner core, freeze fracturing technique was used to section the implant.
5.3.2 Optical / Scanning Electron Microscopy

For the morphological study, 2 groups of gel formulations were prepared. Both groups comprised 20% triacetin and varying concentrations of PLGA and NMP; one group contained 20% PLGA and 60% NMP, whereas the other contained 40% PLGA and 40% NMP. A third group, consisting of single solvent NMP gel formulation, was prepared as a positive comparison for the shell structure and its porosity.
Figure 5-9 shows the cross-sectional images of the 3 gel formulations under electron and optical microscopy. As mentioned earlier on in Section 2.4.2.1, in the single NMP solvent gel system, the shell morphology consisted of numerous large and tear-shaped cavities. These cavities were uniform in shape and size and covered the entire circumference of the section. These cavities exhibited high drug diffusivity compared
with the cavities in the polymer-rich matrix. Consequently, for drug diffusion, these lower resistance pathways allowed the rapid elution of encapsulated peptide during the initial 24 hours. This evidence was consistent with the high peptide burst release observed in the release studies.

For the other gel formulations containing triacetin, the observed shell morphology was slightly different. As the NMP content decreased, the shell structure transitioned from a more porous, highly interconnected pore structure to one that contained smaller and denser structure. The smaller cavities made it more difficult for drug to elute rapidly. Instead, the drug had to diffuse through the lower diffusivity polymer-rich matrix. As such, the initial drug burst release was suppressed. Although there was evidence of large cavities, these were significantly less in number and tended to more irregular in shape.

![Figure 5-10: Demarcation of the Shell Structure](image)
Figure 5-11: Shell Thickness of Various Gel Formulations
* P < 0.05, Compared with Control
Ψ P < 0.05, compared between fixed triacetin (TRI) groups

Figure 5-10 shows the demarcation of the shell thickness, and Figure 5-11 shows the shell thickness measurements based on Figure 5-10. Figure 5-10, shows that the shell structure was significantly thicker in the single NMP solvent gel system compared with the co-solvent systems. With the addition of triacetin to the co-solvent systems, the overall hydrophilicity of the gel solution was altered. As such, co-solvent system containing larger amount of NMP was more hydrophilic, leading to the formation of a thick shell structure.
Chapter 5: *In vitro* Studies: Injectable Gel System Analysis

The shell thickness data from the SEM analysis were consistent with scenario 2 in postulation 2B (Figure 5-8, section 5.2.3). Postulation 2B suggests that a higher gel hydrophilicity leads to a higher L-L separation rate (higher solvent efflux), which, in turn, forms a thicker outer shell. This thicker shell layer helped to impede or retard the rate of drug release. Therefore, in the shell thickness study, the co-solvent gel formulation and either the higher or the lower NMP loading demonstrated similar initial peptide burst release.

### 5.3.3 Water Uptake

The previous sections focused on the importance of phase inversion process. However, as mentioned, the hardening of the implant depends on the water ingress rate. A study was performed to demonstrate the difference in water uptake rate between a single solvent system and a co-solvent system. In this study, the water uptake was compared between the single solvent NMP system and the 40% NMP / 20% triacetin co-solvent system.
Figure 5-12 shows a plot of the swelling ratio between the single hydrophilic solvent system and the co-solvent system. The figure shows that the single solvent gel implant exhibited a faster and higher water uptake compared with the co-solvent gel implant. The use of co-solvent reduced the overall solution hydrophilicity, which led to the slower ingress of water into the gel implants. Typically, the initial water uptake is related to the porosity of the shell structure. The single solvent NMP system demonstrated a faster initial water uptake rate, which led to the formation of numerous tear-shaped pores, whereas the opposite was true of the co-solvent demonstrated a slower water uptake.

5.4 Contact Angle Analysis

Postulation 2B states that a change in phase separation kinetics is a result of changes in gel overall hydrophilicity in co-system systems. Data from the morphological study on
shell thickness demonstrated that the shell thickness decreases with increasing composition of hydrophobic solvent in the gel formulation. This supports scenario 2 in postulation 2B. To further assess the validity of postulation 2B, the hydrophilicity of various gel formulations were analyzed. The hypothesis is that varying composition of hydrophilic and hydrophobic solvents in the gel formulation will change the overall gel formulation hydrophilicity. This change in overall gel hydrophilicity led to a gelling transition from a fast gelling system (hydrophilic NMP single solvent system) to a slow gelling system (increasing amount of hydrophobic triacetin solvent in co-solvent system). This resulted in a change in shell thickness and porosity as explained in section 5.3.2.

Gel hydrophilicity was performed through contact angle measurement. Contact angle of gel formulation changes with its hydrophilicity. The higher the hydrophilicity is, the lower the contact angle. This is due to a phenomenon known as ‘wetting’. A hydrophilic system tends to wet the surface more, resulting in a smaller contact angle.

In this contact angle study, 3 groups of gel formulation were prepared. The 3 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
Ψ P < 0.05, compared between fixed triacetin (TRI) groups

Figure 5-13 shows the measured contact angle and the corresponding shell thickness of the 3 gel formulations. The figure demonstrated that gel formulations consisting of the hydrophobic triacetin has a significantly larger contact angle as compared to the control group which does not contain triacetin. The 2nd and 3rd gel formulations consist of the same amount of hydrophobic triacetin with varying amount of NMP. It is
demonstrated that gel formulation with a higher NMP composition has a smaller contact angle than that with lower NMP composition despite both gel formulation having the same amount of triacetin composition. This data strongly suggest that the higher NMP formulation is more hydrophilic. This shows that the rate of gelation of the gel formulation is directly dependent on the overall gel hydrophilicity. This finding is in line with the shell thickness result and further supports postulation 2B.

5.5 Polymer Degradation Study

The degradation profile of the polymer used in the gel system was characterised using a co-solvent gel system (40% PLGA / 40% NMP / 20% triacetin) compared with a single solvent NMP gel system performed in parallel.
Figure 5-14: Degradation Profile of Single NMP and Co-Solvent Gel System

Figure 5-14 shows the degradation and polymer mass profile. As discussed in earlier sections, the initial drug burst release was highly dependent on the type of shell structure. A fast gelling system produces very porous shell structure, which leads to a high initial drug burst release. However, following the hardening of the implant depot,
the drug release rate shifted towards drug diffusion centric. The rate of drug diffusion is also dependent on the rate of degradation of the polymer used in the gel system.

PLGA 50:50, with an inherent viscosity of 0.2, is a fast-degrading polymer. When coupled with the carboxylic end-group, the overall hydrophilicity of the polymer increases. Consequently, the polymer attracts water and degrades faster. The polymeric degradation of PLGA is an auto-catalytic process. The polymer chains are hydrolysed into acidic oligomers, which reduce the pH of the internal micro-environment to approximately 4. In this case, the degradation profile of the co-solvent gel system did not differ significantly from that of the single NMP gel system.

The overall mass of the polymer matrix remained unchanged for approximately 5 days before the oligomer started leaching out of the depot, which gradually reduced the mass of the remaining polymer matrix. Following the loss of polymer mass after day 5, it was easier for the drug encapsulated within the matrix to diffuse out of the depot. Therefore, as demonstrated by the drug release profiles shown in the previous chapter, the rate of drug release typically started to increase after day 6.
Chapter 6 \textit{In vivo} Studies

For the delivery of CD-NP, the ideal peptide release profile is one that follows a zero-order release profile. As explained, it is crucial to minimize the initial burst release to avoid inducing undesired hypotensive conditions in patients. The subsequent release rate has to fall within a peptide therapeutic window. In this case, the targeted dose was above $10^{-7}$ g/kg/min. This dose has been demonstrated to exert beneficial effects in a myocardial infarction model [170].

Following the extensive \textit{in vitro} studies, a gel formulation was selected based on the desired peptide release profile that best mimicked the ideal profile. This gel formulation consisted of 0.45\% CD-NP and 40\% polymer dissolved in 40\% NMP and 20\% triacetin. Figure 6-1 shows the peptide release profile and the daily peptide release amount in this system.
This gel formulation demonstrated a sustained CD-NP release for more than 30 days, with an initial peptide burst release of 11 ± 1% of the total CD-NP loading, which translated to 76 ± 15.9 μg of CD-NP released within the first 24 hours. Thereafter, the rate of CD-NP release started to decrease and assumed a linear release pattern, ranging from 5.95 to 11.9 ug/day over the next 30 days. Up to 50 ± 1% of the total CD-NP loading was released by day 31.

*In vivo* rat studies (3 in total) were performed to evaluate the efficacy of the gel system. The first 2 studies were performed in normal healthy rats, whereas the third study was performed in the acute myocardial infarction rat model. This model was selected because MI is the most common clinical representation among patients suffering from HF conditions.

### 6.1 Rat Study I (Normal Healthy Rats)
6.1.1 Study Outline

The purpose of this study in healthy rats was to serve as a proof-of-concept (POC) for the delivery of CD-NP in the in situ forming implant system. In this POC study, the gel formulation discussed above was used. Additionally, another gel formulation, which elicited a profile similar to that of the desired peptide release profile, was selected for testing in rats. The blood collection was performed at two time points, 24 hours and 2 weeks. The 24-hour time point was selected to determine the extent of the initial burst release, whereas the 2-week time point was selected to determine the peptide release sustainability of this gel delivery system. The two gel formulations were as follows:

- **Formulation 1 (F1):** 0.45% CD-NP / 40% PLGA 50:50 / 20% Triacetin in NMP
- **Formulation 2 (F2):** 0.45% CD-NP / 40% PLGA 50:50 / 30% Triacetin in NMP

In total, 25 male Wistar rats were randomly assigned into 5 groups of 5 rats each. The rat groups were as follows: Group 1, 24hrs F1 gel treatment; Group 2, 2 week F1 gel treatment; Group 3, 24 hrs F2 gel treatment; Group 4, 2 week F2 gel treatment and Group 5, vehicle group. The rats in this study were maintained on a standard laboratory diet and were allowed at least 3-4 days to acclimatise to the animal facility housing prior to the start of the study.

**Gel Implantation**

To achieve the required peptide dosage of $10^{-7}$ g/kg/min (43.2 ug/day based on the rat body weight), 0.7 cc of F1 gel was injected into each of the F1 gel treatment rats,
whereas 1.0 cc of F2 gel was injected into each of the F2 gel treatment rats. The vehicle group was injected with 0.7 cc of blank F1 gel.

To minimise the suffering of the animals during gel implantation, the rodents were anaesthetised using isoflurane (1.5% in oxygen) and ventilation was provided using a rodent ventilator. To minimise any unnecessary risk of infection, the injection sites (back of the rat) were shaved and swabbed with 70% ethanol prior to the injection. The respective volumes of freshly prepared gel were drawn into a 3-cc syringe and a 20G needle was then attached to the syringe tip. The gel solution was administered through sequential subcutaneous injections into the rodent’s backs. After the injection, the rodents were returned to their cages and were allowed to recover.

6.1.2 Results and Discussion

6.1.2.1 Plasma Evaluation

*Plasma CD-NP concentration*

![Figure 6-2: In vivo rat study I: Plasma CD-NP & cGMP A) Plasma CD-NP B) Plasma cGMP](image)
Figure 6-2 A shows the results of the plasma CD-NP concentration (pCD-NP conc.). The red line represents the desired pCD-NP conc. throughout the duration of the release.

At 24 hours after the gel injection, pCD-NP conc. was maintained at 115 pg/ml for the control group, which was similar to the background level. For the treated groups, pCD-NP conc. increased to 20,000 ± 11,700 pg/ml and 9,000 ± 6,012 pg/ml for formulation 1 and 2, respectively. These levels dropped to 2,600 ± 632 pg/ml and 1,600 ± 503 pg/ml, respectively, at 2 weeks after injection. The initial increase in pCD-NP was expected because of the system’s initial burst. At 2-week time point, the plasma CD-NP level observed was somewhat unexpected. In an unpublished study performed by researchers at Mayo Clinic, a plasma CD-NP steady-state concentration of approximately 5,000 pg/ml was observed for a dosage infusion rate of 43.2 ug/day. The in vitro data suggested that the amount of CD-NP released from the system would be higher than this targeted dosage. Therefore, the pCD-NP was expected to be at least close to or higher than 5,000 pg/ml.

A slower rate of polymer degradation or a diffusion barrier within the subcutaneous (SQ) tissue may have contributed to the observed pCD-NP level. In the SQ physiological environment, the availability of water is limited compared with that of the in vitro study. Because the degradation of the polymer is primarily because of hydrolysis, the limited availability of water might have contributed to the slower degradation rate, leading to the
slower release. In addition to exhibiting a possible slower polymer degradation rate, the week2 results demonstrated that the injectable gel system provided a certain amount of protection against degradation of the drug and suggested that the conformational structure was maintained. The C-NP RIA assay is highly specific and detects CD-NP only if the ring structure within the drug is intact. Therefore, the detection of CD-NP at week 2 suggested that the drug was intact and was in an active form.

**Plasma cGMP Concentration**

To investigate the bioactivity of the released CD-NP, plasma cGMP concentration (pcGMP conc.) was measured. As discussed, cGMP is a second messenger that is activated through the binding action of CD-NP onto NPR-A / B receptors. It has been demonstrated that only an intact ring structure will activate the NPR-A receptor [17, 171]; therefore, the presence of cGMP would certainly confirm the biological activity of released CD-NP.

Figure 6-2 B shows the plasma cGMP concentration (pcGMP conc.). The red line represents the maximum pcGMP conc. in male Wistar rats, as reported by Mayo Clinic. Likewise, the pcGMP conc. in the control group mirrored the systemic background cGMP level. For the treated groups, the pcGMP conc. increased to 220 ± 106 pmol/ml and 280 ± 76 pmol/ml for formulation 1 and 2, respectively, after 24 hrs. After 2 weeks, the pcGMP level decreased to 170 ± 77 pmol/ml and 130 ± 39 pmol/ml for formulation 1 and 2, respectively. The 24-hr pcGMP concentrations for the treated groups were as expected. Given the finite number of receptors available, there is a maximum cGMP
activation level. Therefore, despite the high level of pCD-NP observed at 24 hrs, the cGMP level remained at the maximum output level. Although the 2-week cGMP levels were not statistically significant compared with the control, the absolute mean value demonstrated an increase. The detection of increased cGMP levels in the treatment group demonstrated the bioactivity of the released CD-NP; cGMP is activated only when intact and bioactive CD-NP binds with the NPR A/B receptors. Therefore, the CD-NP must have retained its conformational structure.

6.1.3 Conclusion

This study demonstrated that the burst release from the gel system was high but was tolerated by the rodents. Additionally, sustained CD-NP peptide release was observed for up to 2 weeks. The lower than expected plasma measurements were likely because of slower polymer degradation. This study also demonstrated that the gel exhibited peptide-protecting properties, which was evidence from 1) the detection of CD-NP using the conformation-sensitive CNP RIA kit and 2) the increased cGMP concentration at week 2.

6.2 Rat Study II (Normal Healthy Rats)

6.2.1 Study Outline

This study served as an extension of rat study I. In rat study I, gel formulation 1 produced the higher peptide sustained release at week 2. Therefore, in this study, only gel formulation 1 was used.
The release profile of any injected gel is dependent on the kinetics of phase inversion as well on the rate of polymer matrix degradation. The degradation, in turn, is dependent on intrinsic and extrinsic factors, one of which is the availability of water because the polymer matrix degrades hydrolytically. Studies have shown that in the subcutaneous environment, the relatively limited availability of water means that the polymer matrix tends to degrade at a slower rate compared with in vitro studies [172]. Polymer chain degrades hydrolytically. As such, lowered availability of water in the subcutaneous environment will retard rate of polymeric degradation. This phenomenon was observed in rat study I in which a lower peptide release was detected at week 2. Therefore, instead of injecting the entire gel volume into a single site, the gel sample was divided between several different sites, each receiving 0.2 cc of the formulation. The smaller injection volume should provide a much larger surface area for the polymer degradation.

In addition to the multiple injection site strategy to improve peptide release, a higher gel volume was used to produce a sustained peptide release at week 3. Therefore, in this study, a gel volume of 2.8 cc was administered. Bearing in mind the possibility of side effects from the initial burst release of the gel system, precautions were taken to separate the required 2.8 cc into 2 separate 1.4-cc injections. The injections were performed on day 0 and on day 2 to allow a 48-hour recovery window in which the initial burst effect could dissipate.
In this study, male Wistar rats were randomly assigned into 4 groups of 5 rats each. The groups were as follows: Group 1, 1-week gel treatment; Group 2, 2-week gel treatment; Group 3, 3-week gel treatment and Group 4, vehicle group. The rats in this study were maintained on a standard laboratory diet and were allowed at least 3-4 days to acclimatise 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 rodents were anaesthetised using isoflurane (1.5% in oxygen), and ventilation was provided using a rodent ventilator. To minimise any unnecessary risk of infection, the injection sites (on the back of the rat) were shaved and swabbed with 70% ethanol prior to injection. Aliquots of freshly prepared gel (1.4 cc) were drawn into a 3-cc syringe, and a 20G needle was then attached to the syringe tip. Approximately 0.2 cc of gel was injected subcutaneously into each of 7 sites on the back of the rodent, resulting in 1.4cc of total injected gel volume. After the injection, the rodents were returned to their cages and were allowed to recover. A second injection, an exact repeat of the first injection, was performed 48 hours (day 2) later.

**6.2.2 Results and Discussion**

Without exception, the rats recovered well after each gel injection. Typically, recovery 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 because of the series of events that the rat experienced following the first injection. The rats underwent multiple
anaesthetisations on days 0 and 2 during the gel injection procedure, and between the injections, the rats were subjected to multiple blood pressure measurements. The procedure for blood pressure measurement involved holding the rats in a confined space that was heated to 37°C. This series of events may have been too traumatic for the rat that died.

6.2.2.1 Blood Pressure Measurement

As a non-invasive method to assess the presence of circulating CD-NP levels in the rodents, the blood pressure was measured. Given the veno-dilating property of CD-NP, the presence of any circulating CD-NP will be indicated by a lower blood pressure. Therefore, the blood pressure (BP) was monitored for both the treatment and vehicle group. BP measurements were taken once every 2 - 3 days, and additional readings were taken within 24 hrs after each injection. Figure 6-3 A1 and B1 shows the mean BP trend measured over the 18 days of the study for the treatment and vehicle group, respectively. Figure 6-3 A2 and B2 shows the mean BP trend, magnified over the initial 3 days.
Chapter 6: In Vivo Studies

Figure 6-3: Blood Pressure Fluctuation over 18 days
A1) BP trend for CD-NP treatment groups A2) Magnified 3-day BP trend for treatment group
B1) BP trend for vehicle group B2) Magnified 3-day BP trend for vehicle group
* P < 0.05, Comparison between BP of treatment and vehicle group at the same time point

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

As expected, the mean BP for the CD-NP-treated groups dropped from 102 ± 1.2 mmHg to 66 ± 1.7 mmHg within the first 6 hrs after gel injection 1. This drop in BP
coincided with the initial burst release from the gel system. The BP recovered to approximately 83 ± 1.8 mmHg within the first 24 hours. Following the second injection on day 2, the BP dropped from 86 ± 1.1 mmHg to 50 ± 3.0 mmHg. After the BP recovery from the initial burst effect of the second injection, the mean BP was maintained at between 76.7 mmHg and 90.9 mmHg until the end of the study (day 18). This decrease in the BP was significant compared with the vehicle BP. The BP trend in the CD-NP-treated group confirmed that CD-NP was released continuously from the gel system over the entire 18-day test period and exerted its bioactive vaso-dilatory function. A CD-NP infusion study in normal dogs also demonstrated lowering of mean arterial pressure, and the change in BP was CD-NP-dose dependent [16].

6.2.2.2 Plasma Evaluation

*Plasma CD-NP concentration*

Plasma CD-NP concentration (pCD-NP conc.) was quantified using the radio-immuno assay (RIA) kit. Figure 6-4 A shows the results of the pCD-NP conc. measurements, demonstrating the sustained release of CD-NP over the 3-week study period. The measured pCD-NP conc. was 33,000 ± 2,888 pg/ml, 14,000 ± 3,302 pg/ml and 8,000 ± 1,115 pg/ml at 1, 2 and 3 weeks, respectively. The pCD-NP conc. in the vehicle group was approximately 280 ± 160.4 pg/ml. The data from the CD-NP-treated groups clearly demonstrated an elevated pCD-NP level that was significantly different from the vehicle group.
If the *in vitro* data were representative of the *in vivo* release, one would expect the pCD-NP conc. at the 3-week 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 rate of degradation of the polymer matrix *in vivo* compared to the degradation rate *in vitro*. However, this did not appear to be the case. The slower peptide release was observed in rat study I, and it was postulated that slower degradation was the reason. To circumvent this issue, the gel injected for this study was separated into multiple injection sites. However, this mode of injection did not demonstrate an increase week 3 plasma CD-NP concentrations at week 3.

Second, there could be a loss of CD-NP bioactivity. As discussed, the CNP RIA kit only detects NPs with intact ring structures. As the PLGA matrix degrades into lactic and
glycolic acids, it creates pockets of slightly acidic environments in the polymer matrix [172]. This internal acidic environment might have degraded the fragile CD-NP peptide. Because the RIA kit only detects intact CD-NP, the resulting pCD-NP conc. could be lower than expected.

Third, the gel implant was injected subcutaneously. Because peptide diffusion had to dissipate from the site of the gel implant all the way to the blood stream, a large portion of the residual peptide may have remained in the tissue, effectively lowering the amount of peptide that entered the blood stream and therefore lowering the plasma concentration of the peptide. This scenario will be discussed further in chapter 7.

**Plasma cGMP Concentration**

The pcGMP conc. results shown in figure 6-4 B demonstrate that the pcGMP conc. was 270 ± 41.4 pmol/ml, 190 ± 48.1 pmol/ml and 114 ± 15.8 pmol/ml at the 1-, 2- and 3-week time points. The pcGMP conc. in the vehicle group was approximately 108 ± 15.5 pmol/ml. Although pcGMP conc. was elevated significantly at week 1 compared with the vehicle group, a less significant difference was observed at week 2 and week 3.

The plasma cGMP concentration is dependent on the amount of pCD-NP and the availability of NPR-A / B receptors. Studies have demonstrated that CD-NP activation of cGMP is dose dependent, and this relationship plateaus at a high CD-NP conc. [16, 17]. Based on unpublished data from previous studies (Burnett et al.), it was determined that the maximum pcGMP conc. in this particular rodent species is between 250 and 300
pmol/ml. It was also determined that a pCD-NP conc. of 5,000 pg/ml is sufficient to achieve the maximum pcGMP conc. Given the high level of pCD-NP administered, it was expected that the maximum activation of cGMP would be sustained throughout the 3-week period; however, this did not occur.

There is a possible explanation for this observation. In this pilot study, the objective was to achieve the sustained release of CD-NP from the gel system; therefore, an excess of CD-NP was loaded into the depot, and a high level of pCD-NP in plasma was achieved, even at 3 weeks. The abundant availability of pCD-NP may have led to the continuous maximal activation of cGMP over an extended period. This high cGMP activation rate may not have been perceived as ‘normal’ by the body. Therefore, compensatory responses might have intervened to regulate this abnormally high amount of pcGMP. These responses will be discussed in detail in the following section.

6.2.2.3 Urinary Output Evaluation

**Urinary Output Volume**

Table 6-1 shows the 24 hrs urinary output for both the treated and vehicle rodents. The mean 24 hrs baseline urine output for the treated and vehicle group was 15 ± 1.3 ml and 13 ± 0.9 ml, respectively. After the first and second gel injection in the treatment group, the mean urinary output increased to 32 ± 2.5 ml and 32 ± 1.8 ml, respectively. The increase in urinary volume output was approximately twice that of the baseline volume. CD-NP is known to be diuretic [16, 17]; therefore, the increase in the urinary output directly after the injections may be related to the amount of CD-NP administered.
During the first 24 hours after each injection. This is further evidence that CD-NP released from the gel polymer matrix was biologically active.

### Table 6-1: 24 hours Urinary Output Volume
(Baseline, 1st & 2nd post-injection, prior to sacrificial)[173]

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Treatment Group / mL</th>
<th>Vehicle Group / mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>After Injection 1</td>
<td>32.4</td>
<td></td>
</tr>
<tr>
<td>After Injection 2</td>
<td>31.5</td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>2 weeks</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>3 weeks</td>
<td>12.5</td>
<td>11.9</td>
</tr>
</tbody>
</table>

**Urinary CD-NP and cGMP**

The background urinary CD-NP (uCD-NP) level, shown in figure 6-5, was $2 \pm 0.10$ pg/min and $4 \pm 1.65$ pg/min for treatment and vehicle groups, respectively. For the treatment group, the uCD-NP was $550 \pm 139.60$ pg/min and $600 \pm 151.90$ pg/min after the 1<sup>st</sup> and 2<sup>nd</sup> injections respectively. Immediately after the gel injections, there was a burst release of CD-NP from the gel. Most of this unused CD-NP was excreted out of the system through urinary excretion, which accounted for the high uCD-NP output. The amount of CD-NP excreted at 1, 2 and 3 weeks decreased to $107 \pm 12.70$ pg/min, $34 \pm 29.72$ pg/min and $17 \pm 8.19$ pg/min, respectively. These decreases were statistically significant compared with the baseline levels. As mentioned, following the burst release, the amount of CD-NP released from the gel system was reduced to a more sustainable
level, which in turn led to the lower uCD-NP output observed. There was no significant difference in uCD-NP output observed between the baseline and urinary output at 3 weeks for the vehicle group.

Figure 6-5: 24 hours Urinary CD-NP Output
A) Treatment groups B) Vehicle Group
* p < 0.05, Treatment vs. Baseline[173]

Figure 6-6: 24 hours Urinary cGMP Output
A) Treatment Groups B) Vehicle Group
* p < 0.05, Treatment vs. Baseline[173]
Figure 6-6 shows that the baseline urinary cGMP (ucGMP) level was 29 ± 0.10 pmol/min and 31 ± 2.68 pmol/min for treatment and vehicle group, respectively. There was no significant change in the ucGMP output for the vehicle group at 3 weeks after the injection of the blank gel. The ucGMP output in the treatment groups increased significantly following the gel injections. The ucGMP output was 41 ± 2.16 pmol/min and 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. This is likely because there was a lapse time between the CD-NP diffusing out of the gel system into the blood, the binding of CD-NP with NPR-A / B receptors, the activation of the cGMP and the filtration of pcGMP in the kidney. UcGMP output remained high at 1 and 2 weeks. However, the output dropped to a level that was not significant at 3 weeks after the treatment. An interesting observation was that the ucGMP output level was consistently high at weeks 1 and 2.

Earlier in this study, blood pressure, plasma CD-NP and cGMP and urinary CD-NP and cGMP were discussed. The gel system demonstrated an elevated pCD-NP level throughout the 3-week treatment period. The high level of uCD-NP recorded was consistent with the elevated pCD-NP. The bioactivity of released CD-NP was also retained, as demonstrated by the initial elevation of the pcGMP level at week 1 and by the sustained hypotensive state of the treated rats.
However, in the long-term, pcGMP level did not maintain the saturation level that was predicted by the level of pCD-NP. Following the pcGMP saturation level at week 1, pcGMP decreased gradually over the next 2 weeks to a level that was not significantly different from baseline. Evidence from ucGMP excretion suggested that the decrease in pcGMP was not because of the loss of bioactivity of released CD-NP. Despite the decreasing pcGMP level between week 1 and week 3, a constantly high level of ucGMP was recorded for up to 2 weeks into the treatment. Instead, it is believed that following the initial days of saturated cGMP production, the body may have perceived the level as ‘abnormal’. Therefore, compensatory responses may have intervened to regulate this abnormally high level of circulating pcGMP. The two possible mechanisms of 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 activation rate. In this mechanism, the NPR-A / B receptors in the natriuretic peptide / cGMP pathway are regulated, preventing further binding of CD-NP to the receptor. NPR-C receptors, also known as the NP clearance receptors, go into over-drive, increasing the removal rate of circulating CD-NP, which helps reduce the pCD-NP concentration and in turn, reduces the availability of CD-NP for binding NPR-A / B receptors. This increased pCD-NP clearance was confirmed by the elevated uCD-NP output. In addition to the NP/cGMP pathway, there is another cGMP pathway, known as NO-sGC/cGMP pathway. This
pathway also promotes cGMP production and can be down regulated to reduce further the cGMP production [174, 175].

The second mechanism involves reducing the circulating pcGMP level, which is achieved by increasing the cGMP removal rate through degradation of cGMP by phosphodiesterases (PDE), such as PDE V, or more directly through urinary excretion.

Based on the high level of sustained ucGMP output recorded at week 2, it is very likely that the circulating pcGMP conc. was lower than expected because of the high urinary excretion rate. Therefore, this accounts for the gradual drop in the pcGMP level between week 1 and week 3.

6.2.3 Conclusion

This study demonstrated that with the appropriate gel formulation, CD-NP release was sustained over a 3-week period. The bioactivity of released CD-NP was maintained for up to 3 weeks, although not at the levels expected from in vitro data. This pilot study also demonstrated that with a CD-NP delivery system, it might not be necessary to sustain a high level of CD-NP release. The key is to determine the right dosage of CD-NP that is needed to stimulate a slightly elevated but not excessive amount of cGMP.

However, there were some limitations to this in vivo study. The bioactivity marker of released CD-NP was based on cGMP level. Antagonistic systems come into play in an in vivo setting; therefore, it might be necessary to test for other biological markers to
assess further the actual influence of these systems. Additionally, it is possible that the gel system could be used in disease models to potentiate the efficacy of the system.

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 selected to investigate further the efficacy of the gel system because it is the most common clinical representation of HF conditions. The high pCD-NP level observed over the 3-week period in the predicate study demonstrated that the volume of the gel injection could be reduced further. Therefore, for this AMI model rat study, the gel injection volume was reduced from 2.8 cc to 2.0 cc. The strategy to mitigate the harmful side-effects of the initial peptide burst release was continued in this model by dividing the 2.0-cc targeted volume into 2 x 1.0 cc injections. The 1.0 cc injections were sub-divided into 5 locations, with approximately 0.2 cc injected per site.

Male Wistar rats were randomly assigned into 2 groups of 15 rats each. Rat groups were as follows: Group 1, vehicle group (gel only) and Group 2, treatment group (gel and CD-NP). The rats used in this study were maintained on a standard laboratory diet and were allowed at least 3 - 4 days to acclimatise to the animal facility housing prior to the start of the study. In this study, 15 rats were used per group to allow for the possibility of mortality sustained from the model-creation surgical procedure and/or the unsuccessful model creation. The study protocol involved injecting the rats with 1.0 cc of gel solution on day 0, followed by the model creation surgical procedure on day 1 and
finally, a second 1.0-cc injection was administered on day 3. The rats were sacrificed at week 3 for data collection.

**Gel Implantation**

Similar to the predicate study, the animals were anaesthetised using isoflurane (1.5% in oxygen) and ventilation was provided using a rodent ventilator. The injection locations were swabbed with 70% ethanol prior to injection. After the first set of injections, the rodents were returned to their cages and were allowed to recover. After 72 hours, a second injection was administered using the same procedure as the first injection but at different sites.

6.3.2 *Acute Myocardial Infarction Model Creation*

Following the first set of gel injections, the rats were retrieved for the creation of the infarction. Briefly, rats were anaesthetised as described in previous sections. The chest hair was shaved and the chest was swabbed with 70% ethanol prior to dissection [176]. The chest was opened using an intercostal incision and was retracted using a rat rib retractor. The left auricle was retracted slightly to expose the entire left main artery system. Ligation, using a tapered forceps, was performed by tying a 7-0 suture onto the anterior descending coronary artery (LAD). The area immediately beneath the ligation was examined briefly to confirm the presence of ischemia. A dose of lidocaine was administered over the heart to reactivate the heart rhythm. The chest wall was closed using a 5-0 Ticron blue polyester fibre suture with one layer through the chest wall and muscle. To minimise any inflammation, before suturing the second layer through the
skin, several drops of hydrogen peroxide were added. The rats were removed from the ventilator and were kept warm using a heating pad. Buprenex (0.07 cc) was injected into the blood vessel to aid the recovery process.

### 6.3.3 Results and Discussion

In total, 30 rats were used in the study. However, 4 rats in each group did not survive the ligation surgery for the AMI model creation; the deaths resulted from a punctured heart during LAD ligation, massive internal bleeding or were related to the surgical procedure. In addition to the recorded deaths, 1 rat from the treatment group (gel + CD-NP) and 3 rats from the vehicle group (gel only) were excluded from the data computation because of a poorly defined infarction or unsuccessful AMI creation. No deaths were recorded because of gel injections. The statistical data used in this discussion were based on 8 and 10 rats from vehicle and treatment groups, respectively.
6.3.3.1 Blood Pressure Measurement

The predicate study performed by our group demonstrated that the BP was maintained at a lowered level throughout the study duration [177], providing evidence that CD-NP was eluted consistently out of the gel and demonstrated bio-activity.

In this AMI model rat study, BP was measured before the start of the study and before sacrificing the rats. For additional data collection, BP was also monitored immediately after each injection to determine whether the BP dropped as a direct result of the gel initial burst release.
Figure 6-8 shows the BP data collected at the pre-determined time points. No BP change was observed in the vehicle group following the two gel injections. However, at the 3-week time point, BP 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 1\textsuperscript{st} and 2\textsuperscript{nd} gel injections, respectively. The BP at 3 weeks was 79 ± 2.0 mmHg.

The following three observations were made from the BP measurement data:

First, in this study, BP of the treatment group was 73 ± 1.1 mmHg and 69 ± 2.3 mmHg following the 1\textsuperscript{st} and 2\textsuperscript{nd} gel injections, respectively. Compared with the predicate study in which the BP of the treatment group dropped to 66 ± 1.7 mmHg and 50 ± 3.0 mmHg.
after the two injections, the drop in the BP for this study after the 1\textsuperscript{st} and 2\textsuperscript{nd} injection was less dramatic. The observation of rat activity level post-injection suggested that the rodents tolerated this drop in BP better than the more drastic drop observed in the predicate study. This difference in the BP drop was because of the drug dosage given in this study (1.0 cc per injection), which was lower than in the predicate study (1.4 cc per injection). As explained, the initial drug burst release was expected from the gel system, and this sudden elevation in plasma CD-NP resulted in the BP drop. The reduction of the amount of drug in the initial burst (due partly to the lower loading) resulted in a more acceptable BP drop.

Second, the BP at 3 weeks in the treatment group was 79 ± 2.0 mmHg. This lowered BP level (compared to baseline) suggested that the gel was eluting biologically active CD-NP even at 3 weeks. This observation was consistent with the predicate study.

Third, the BP in the vehicle group remained unchanged following the two injections. However, as the infarction spread and worsened, the heart function decreased drastically, leading to a huge drop in BP from 100 ± 1.9 mmHg to 56.6 ± 0.8 mmHg at 3 weeks. Interestingly, the BP of treatment group at the same time point did not exhibit such an extensive drop in BP. Instead, the BP hovered at 79 ± 2.0 mmHg, demonstrating further evidence of attenuation of the infarction effects in CD-NP-treated group.

6.3.3.2 Plasma Evaluation
The plasma CD-NP concentration (pCD-NP conc.) and cGMP concentration (pcGMP conc.) was quantified using the radio-immunoassay (RIA) kit. Data collected are shown in Figure 6-9. The pCD-NP conc. in the vehicle group was 120 ± 6.2 pg/ml, whereas that of the treatment group was elevated significantly to 5,000 ± 505 pg/ml at 3 weeks.

The pCD-NP measurements from this rat study were consistent with the data from the predicate study. In the previous study, the administration of 2.8 cc of gel yielded 8,000 ± 1,115 pg/ml pCD-NP conc. at 3 weeks, which translated into approximately 2,800 pg/ml per 1 cc of gel. In this study, 2.0 cc of injected gel yielded a pCD-NP conc. of 5,000 ± 505 pg/ml, which translated into approximately 2,500 pg/ml per 1 cc of gel administered. These data affirmed that the gel release was consistent between the two studies.

The pcGMP conc. in vehicle group was 112 ± 8.0 pmol/ml, which correlated with the baseline pcGMP conc. of 108 ± 15.5 pmol/ml in the normal rats. However, the treatment group demonstrated a significantly elevated pcGMP conc. of 170 ± 21.4 pmol/ml at 3 weeks.
weeks. This increased difference between the treatment and vehicle group demonstrated that the gel delivery system preserved the bioactivity of the released CD-NP for up to 3 weeks.

In the predicate study, an excess of CD-NP was injected into rats to demonstrate the sustained release of CD-NP over a 3-week period [177]. At the 3-week time point, the pcGMP concentration was reduced to a level that did not differ significantly from baseline, despite the elevated pCD-NP conc. of $8,000 \pm 1,115$ pg/ml. It was postulated that the sustained delivery of high CD-NP concentrations from the gel led to the consistently high cGMP activation rate. This high activation rate may have been recognised as ‘abnormal’ by the body. Therefore, the drop in plasma cGMP was because of compensatory responses that intervened to regulate the abnormally high amount of pcGMP. The three compensatory responses identified were as follows: first, down regulation of NPR-A / B receptors to reduce cGMP production; second, ‘over-drive’ of NPR-C receptor clearance may have helped to reduce the pCD-NP level and therefore the reduction in cGMP activation; third, the pcGMP level was lowered through the increase in cGMP excretion via kidney filtration. This third mechanism of reducing the plasma cGMP was confirmed by the elevated urinary excretion of cGMP.

The plasma CD-NP and cGMP data from this study supported the above postulation. In this study, at 3 weeks, the plasma CD-NP and cGMP concentrations were $5,000 \pm 505$ pg/ml and $170 \pm 21.4$ pmol/ml, respectively, whereas the 3-week plasma CD-NP and cGMP concentrations in the previous study were $8,000 \pm 1,115$ pg/ml and $110 \pm 35.4$
pmol/ml, respectively. Comparing the two sets of data, a higher sustained CD-NP release (as demonstrated by the elevated CD-NP level of ~8,000 pg/mL) yielded a lower pcGMP conc., whereas a lowered CD-NP release (this study) yielded a higher pcGMP conc. at 3 weeks. In this case, because of the reduced dosage, the suppression of cGMP activation was not as extensive, resulting in a higher pcGMP conc. at 3 weeks.

6.3.3.3 Urinary Output Evaluation

Figure 6-10 shows the urinary CD-NP and cGMP outputs at 3 weeks. The baseline and vehicle group urinary CD-NP outputs were similar, 2.0 ± 0.23 pg/min and 2.1 ± 0.14 pg/min, respectively. As expected, the urinary CD-NP output for the treatment group was significantly different at 120 ± 29.8 pg/min. The high urinary CD-NP output suggested that at 3 weeks, the CD-NP released from the gel was still in excess of what the body needed.

![Figure 6-10: 24 hours Urinary Output](image)

A) CD-NP Output  B) cGMP Output

*P < 0.05 vs. Baseline, † P < 0.05 vs. vehicle
The baseline, vehicle and treatment group urinary cGMP output was 23 ± 1.2 pmol/min, 36 ± 2.2 pmol/min and 52 ± 4.1 pmol/min, respectively. In both the vehicle and treatment groups, the urinary cGMP outputs were elevated significantly compared with the baseline. Furthermore, the urinary cGMP output was significantly higher in the treatment group than in the vehicle group.

The elevated urinary excretion of both CD-NP and cGMP suggested that the drug loading could potentially be reduced even further, provided that this did not affect the 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
The cardiac function and structure of the vehicle and treatment groups are shown in Figure 6-11. Left ventricular ejection fraction, assessed by conventional echocardiogram, was significantly different at 43 ± 3.7% and 59 ± 4.2% for vehicle and treatment group, respectively. The heart / body weight ratio between vehicle and treatment group was also significantly different at 0.337 ± 0.007 and 0.312 ± 0.008, respectively. More important, a significant reduction in fibrosis and collagen deposition was observed in the treatment group compared with the vehicle group. Picrosirius red staining in vehicle group was 1.7 ± 0.3%, compared to 1.0 ± 0.1% for CD-NP-treated group. Figure 6-12 shows the results of the picrosirius red staining.
Figure 6-13 shows the extent of heart infarctions. The extent of the infarction areas between treatment and vehicle group correlated with the data from the ejection fraction and heart / body weight ratio. Figure 6-13 shows that the area of the heart infarction was larger in the vehicle group compared with the treatment group. In addition, the infarcted area in the treatment group appeared more superficial. Similarly, the treated hearts exhibited LV-wall thinning to a lesser extent than hearts from the vehicle group. The percentage difference between the right and left wall of the LV for a normal rat heart is measured at 89%. Treated group showed a reduction of LV wall to 78% while vehicle group had the thinnest LV wall of 31% difference.
The deterioration of both cardiac hemodynamic function and cardiac fibrosis is associated with cardiac remodelling. In this case, the cardiac remodelling was initiated with the ligation of LAD at the start of the study.

Data from the vehicle group demonstrated a marked decrease in heart function, evident from the reduced ejection fraction and hypotensive state experienced by the rats. In addition to the cardiac functions, a higher heart / body weight ratio, the presence of marked fibrosis and collagen deposition and the marked thinning of the LV wall demonstrated the remodelling mechanism in acute myocardial infarction. In contrast, CD-NP-treated group performed significantly better in most aspects of the analysis, demonstrating clearly the cardio-protective effects of CD-NP. A recent study using chronic subcutaneous delivery of CD-NP in rats yielded similar results [178].

The potency of CNP for suppression of collagen production by fibroblasts and the inhibition of fibroblast proliferation is well documented [170, 179]. However, CNP lacks the aldosterone-suppressing property, which is associated with ANP and BNP. The aldosterone inhibitory property of ANP and BNP delays the onset of heart failure and improves cardiac functions. CD-NP, which was developed to attenuate the remodelling process in acute heart failure, exhibits both the anti-fibrotic properties of CNP and the RAAS-suppressing properties of ANP / BNP. Therefore, the combined suppressive effect makes CD-NP an attractive therapeutic peptide for attenuating the onset of cardiac remodelling.
In this novel CD-NP delivery system, the constant release of CD-NP from the gel depot over the 3-week period demonstrated a distinct improvement in both the functionality and structural integrity of the heart. Both systolic and diastolic functions demonstrated better cardiac function, reflected by the improved ejection fraction. Although the ejection fraction was not preserved completely, an improvement was observed. This improvement was also demonstrated by the significantly higher BP compared with the vehicle at week 3. Structurally, the remodelling process was also decreased, demonstrated by the heart / body weight ratio in the treated rats, which was significantly lower compared with the untreated rats. This observation was also corroborated by the reduction in fibrosis and collagen deposition.

6.3.4 Conclusion

The gel delivery system sustained the release of CD-NP over a 3-week period while maintaining peptide biological functionality. The heart remodelling, initiated with LAD ligation, was attenuated, as indicated by the improved cardiac functions and structure in the CD-NP treatment group compared with the vehicle group. The use of one gel injection allowed the system to mitigate the inconvenience of needing to administer repeatedly the therapeutic peptide intravenously. Indeed, this in situ polymer precipitation delivery system for the delivery of the novel chimeric CD-NP is an 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. An in vitro in vivo correlation (IVIVC) is defined by the US FDA as a predictive mathematical model describing the relationship between the in vitro property of an oral dosage form of a drug and the relevant in vivo response. Typically, the in vitro property refers to the rate or extent of drug dissolution or release, whereas the in vivo response refers to the plasma drug concentration or amount absorbed.

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

**Level A**

This level of correlation is the best method for establishing an accurate depiction of the true IVIVC. Level A correlation involves a point-to-point correlation between the entire in vitro and in vivo profiles. The establishment of a level A IVIVC allows the prediction of the entire in vivo drug concentration-time course from the in vitro dissolution profile.

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

\textit{Level C}

This level of correlation establishes a correlation between a single \textit{in vitro} time point and a mean \textit{in vivo} parameter, such as AUC, $t_{\text{max}}$ or $C_{\text{max}}$. This level is generally considered the least accurate of the first 3 IVIVC level classifications. Often, a multiple level C correlation is preferred. In this multiple level C correlation, a comparison is made between several \textit{in vitro} time points and the mean of each associated \textit{in vivo} parameter. The multiple level C correlation generally consists of at least 3 dissolution time points, making it more accurate compared with the level C correlation.

\textit{Level D}

This level of correlation is not considered a formal correlation. The method involves a rank order and semi-quantitative correlation and is generally not useful for regulatory purposes. However, this level of correlation is widely used in preliminary IVIVC of early products in the conceptualisation stage. The IVIVC correlation is easier to establish and serves as an aid in the development of a formulation or processing procedure.

\textbf{7.2 Injectable Gel IVIVC}
Because of the limited number of \textit{in vivo} collection time points, a proper level A, B or C IVIVC as described in section 7.1 was not possible. In this case, a level D correlation was used.

A diffusion model was used to obtain a reasonable correlation between the \textit{in vitro} and \textit{in vivo} data. To simplify the calculation and to keep within known parameters obtained from the \textit{in vivo} studies, the more accurate multi-compartment model was simplified into a single compartment model. The simplified model is shown in Figure 7-1.

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{simplified_diffusion_model}
\caption{Simplified Diffusion Model}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{simplified_diffusion_model}
\caption{Simplified Diffusion Model}
\end{figure}

Where $V_d$ is Drug volume of Distribution and $C_{ss}$ is Drug Steady-State Concentration

This single compartment diffusion model uses the drug infusion rate ($R_i$) and the drug elimination rate ($K_{el}$). The plasma steady-state concentration is represented by the following equation:

\begin{equation}
C_{ss} = \frac{R_i}{V_d \times K_{el}}
\end{equation}

$R_i$ is the amount of drug expected to be released from the system at any point in time. $K_{el}$ is related to $t_{1/2}$ by the following equation (assuming a zero-order law):
In this case, the effects of drug transportation within the tissue, from the tissue into the blood stream and the drug equilibration in the tissue were regarded as negligible.

In an unpublished infusion study performed by researchers at Mayo Clinic, a plasma CD-NP steady-state concentration of 5,000 pg/ml was obtained with an infusion rate of 43.2 ug/day. Using this result and the steady-state concentration equation, the \((V_d \times K_{el})\) value was calculated. In this case, CD-NP \((V_d \times K_{el})\) value in Wistar rats was 8,640.

Using this calculated \((V_d \times K_{el})\) value and the \textit{in vitro} results, the expected plasma steady-state drug concentrations for rat studies I, II and III were calculated. A compilation of the plasma CD-NP \(C_{ss}\) calculations is presented in Table 7-1.
The calculated plasma $C_{ss}$ values were based on the expected rate of the *in vitro* drug release at the respective time points, whereas the measured plasma $C_{ss}$ were derived directly from the *in vivo* results.

With the exception of week 1 time point in rat study II, other plasma $C_{ss}$ values were lower than the calculated value. In rat study I, the percentage difference between the measured and calculated plasma $C_{ss}$ was -31.32% and -183.84% at 24 hours and week 2, respectively. Similarly, the other week 2 and week 3 time points differed by more than -300%. As mentioned previously, several factors, such as drug diffusion and drug equilibration in the tissue, were neglected in the calculation. Therefore, it was reasoned that the measured drug concentrations were lower than the calculated ones.

**Importance of the implant shape and size**
In the *in vitro* studies, the shapes of the injected implants were generally spherical. However, the *in vivo* injected gel did not always result in a spherical implant. Because the gel was injected subcutaneously and the surface tension of the skin tends to depress and spread out the gel significantly during the shell structure hardening process, the shape of the *in vitro* and *in vivo* implants could differ. Therefore, in the case of the 24 hrs time point in rat study I, the measured drug concentration was relatively higher and closer to the calculated values compared with other time points. This was likely because of the increase in the implant surface area, which promoted a faster drug release during the initial 24 hrs.

Interestingly, the week1 time point in rat study II also demonstrated a higher plasma concentration, which could simply be because of the time taken for the shell structure to harden. In the co-solvent gel system, it could have taken up to 1 week before all the solvents in the implant were discharged. Consequently, the implant was maintained in a semi-fluid condition, which allowed the peptide to continue to elute at a faster rate over a number of days following the initial 24 hours. As such, the higher sustained burst lasted for a number of days. In this study, in addition to the first gel injection on day 0, another set of injections were performed on day 3. The combined prolonged burst release from the two sets of injections, coupled with the changes in implant shape (described above), resulted in the higher than expected plasma concentration.

*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 rat study I, a single injection of 0.7 cc was administered, whereas in the other two studies, multiple 0.2-cc injections were administered. At the week2 time point in rat studies I and II, the former exhibited a -183.84% difference, whereas the latter exhibited a -332.83% difference. In contrast, at the week3 time point, the percentage differences were similar in rat study II and III. This observation emphasised the importance of understanding the injected volume and the resultant drug release profile.

Figure 7-2: IVIVC Plot of Measured against Calculated CD-NP Plasma Steady-State Concentration
A) Inclusive of initial burst time point B) Exclusive of initial burst time point

Figure 7-2 shows plots of the measured CD-NP plasma steady-state concentration against the calculated values. Figure 7-1 A shows the plot of the initial burst effect of the implant, i.e., at 24 hours and week 1. As discussed, these two time points exhibited a higher plasma concentration because of the change in implant size and the delayed onset of shell structure hardening in the co-solvent system. When these two time points
were removed (Figure 7-2 B), correlation analysis showed that there is a significant correlation between measured and calculated plasma steady state concentration. This relationship followed a linear regression trend line with an $R^2$ value of 0.9923. In this regression equation, the ideal gradient is 1, with an equation coefficient of 0, which indicates a linear relationship. The data demonstrated that the calculated plasma concentration was typically 2.5x higher than that of the actual measured value. Although certain differences between the *in vitro* and *in vivo* measurements were expected, the increased difference was largely because of the simplified single compartment diffusion model that was used in the analysis. A multi-compartment model would certainly improve the accuracy of this relationship. However, the end points and parameters included in the *in vivo* studies did not allow for such detailed measurements; therefore, it was critical that these parameters be taken into consideration when calculating the expected $C_{ss}$. To achieve this, a pharmacokinetic study is recommended for future studies.

Nonetheless, the assumed IVIV relationship (IVIVR) *in vitro* and *in vivo* did clarify some keys points. First, the current IVIVR of 2.5X could be improved by using a multi-compartment model. Second, this diffusion model only holds for hardened implants in which drug diffusion is the primary rate-determining step. Third, to better model the initial burst, other non-linear models, such as Sigmoid, Weibull, Higuchi or Hixson-Crowell, could be used, in which case, the collection time points should be closer together for the earlier phase of the *in vivo* study.
8.1 Conclusion

8.1.1 In vitro Studies: Drug/Solvent Analysis

In this in vitro chapter, drug and solvent method of quantification were developed and established. Micro-BCA protein assay was shown to be appropriate for the quantification of CD-NP in buffer solution. Peptide concentrations ranging from 0.5 ug/ml to 20 ug/ml were demonstrated to form a linear relationship with the absorbance.

HPLC was used to quantify NMP and triacetin. A suitable HPLC method was developed to resolve the buffer, NMP and triacetin peaks. The NMP and triacetin peaks exhibited a retention time of 3.66 mins and 4.642 mins, respectively. NMP was also found to be stable in buffer for up to 6 days. The linear relationship between NMP concentration and HPLC absorbance was established for NMP concentrations ranging from 0.5 ug/ml to 250 ug/ml. An NMP concentration above 250 ug/ml exhibited a non-linear relationship. Triacetin, however, was shown to be unstable in buffer, and the experimental half-life of the solvent was established at 8.78 ± 1.01 days. The linear relationship between triacetin concentration and HPLC absorbance was established for triacetin concentration ranging from 5 ug/ml to 10,000 ug/ml; 5 ug/ml was the lowest HPLC detection limit for triacetin.
Co-solvent homogeneity and stability was also investigated. Both NMP and triacetin exhibited good miscibility without significant solvent partitioning. The HPLC determination of triacetin concentration was independent of the presence of NMP. However, the NMP concentration appeared to be 5 - 10% lower than expected in the presence of triacetin. This 5 – 10% reduction was independent of triacetin concentration.

8.1.2 In vitro Studies: Injectable Gel System Analysis

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

Solvent efflux was also investigated, and it was established that solvent efflux of hydrophilic NMP did not affect the amount of the initial drug burst. The affinity between
NMP and triacetin solvents was also insignificant in the co-solvent gel system. More important, the morphological analysis demonstrated that the thickness of the shell structure changed with the overall hydrophilicity of the gel solution. The shell structure thickness increased when gel hydrophilicity increased. This finding supports the postulation that a similar initial drug burst could be obtained using different gel hydrophilicities. The data from the *in vitro* studies corroborated this postulation.

### 8.1.3 *In vivo* Studies

In total, 3 *in vivo* rat studies were performed. The first rat study in healthy normal rats established three key findings for the gel system. First, this study established that physiologically, the rats could withstand the hypotensive state induced by the initial drug burst release. Second, the polymer degradation was slower in the subcutaneous environment compared with the *in vitro* environment. Last, this study corroborated the maximum CGMP activation rate and its plasma steady state concentration at 250 PMOL/ML.

The second normal rat study demonstrated that the bioactivity of the drug released from the gel delivery system was preserved. This was demonstrated by the sustained hypotensive state experienced by the drug-treated group throughout the 3-week test period. The presence of elevated plasma CD-NP levels throughout the 3 weeks also supported the preservation of peptide bioactivity. Despite the elevated drug release
throughout the 3 weeks, the cGMP activation was not significantly increased. This result demonstrated an interesting yet compelling insight into the homeostasis regulatory system in the body. cGMP was activated throughout the study duration. It is possible that the high cGMP activation rate was deemed abnormal by the body, which then regulated the cGMP level by reducing the rate of cGMP activation and increasing the cGMP excretion rate. The sustained and elevated cGMP excretion was demonstrated in the 24 hr urinary analysis.

The final *in vivo* study was performed in the diseased model. The acute myocardial infarction model was created by ligating the LV at the start of the study. A sustained hypotensive state and an elevated plasma peptide and cGMP level were recorded for the drug-treated group. The levels of these parameters differed significantly from the control vehicle group. The urinary excretion at 3 weeks demonstrated an increase in the CD-NP and cGMP levels, suggesting further the possibility of dosage reduction. More important, the distinct improvements in the functionality and structural integrity of the heart were reported in the gel-treated group. The cardiac function was characterised by left ventricular ejection fraction, whereas cardiac structure evaluations were characterised by the heart / body weight ratio and degree of fibrosis. The visual analysis of LV wall thickness was performed post-sacrifice and was used to assess the degree of the infarcted area and the thinning of the LV wall. The data from this diseased model rat study suggested that the use of the gel delivery system is an attractive approach for the delivery of the novel chimeric CD-NP to improve both patient compliance and quality of life.
8.1.4 In vitro – In vivo Correlation

In the IVIVC analysis, a correlation was established between *in vitro* and *in vivo* data. Because of the limited number of time points collected in the *in vivo* studies, a complete IVIVC was not possible. Instead, the relationship was established using a simplified single 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 the *in vivo* studies. The only exceptions were at the 24 hours and 1 week time points. This observation demonstrated 2 key findings. First, the shape and the size of the implanted gel played an important role in determining the amount of the initial drug burst. This was especially true during the initial few days when the polymer depot was undergoing the hardening process. Second, following the hardening of the polymer depot, the drug release continued at a more consistent rate after equilibrium. The 2.5x difference may have been because of peptide saturation in tissue and peptide diffusion from the gel into the blood vessels, factors that were not accounted for in the simplified diffusion model.

8.2 Recommendations
8.2.1 Gel Ageing Study

In the reported *in vitro* and *in vivo* studies, fresh gel solutions were always prepared 1 day in advance of the study to minimise variability in the results caused by the ageing of the gel sample. From a commercial viewpoint, it makes practical sense to understand the stability of this gel solution and the ageing effect. The effect of ageing in the gel solution and its resultant release characteristics have been investigated previously [88]. It was reported that polymer network formations typically form in aged gel samples. These formations tend to increase over time. However, the formation of these structures was found to be thermoreversible. Upon heating or injection of the sample into buffer solution, the network formation disappeared. Therefore, it would be useful to understand how ageing affects the release characteristics in this particular co-solvent gel system.

8.2.2 Detailed *In vitro – In vivo* Correlation Study

A main limitation of the IVIVC discussed in this work was the lack of sufficient time points, especially during the earlier phase of the study. For a proper and more detailed IVIVC to be modelled over a non-linear model, such as Sigmoid, Weibull or Hixson-Crowell models, the *in vivo* study must be re-designed to include collection time points that are closer together. This is especially so for the initial 24 hrs. To improve the mathematical model, the gel samples and the tissue surrounding should be retrieved for residual drug analysis. This will provide a more accurate indication of the amount of actual eluted peptide and the amount of residual peptide in the surrounding tissue.
With the depot residual testing, an *in vivo* cumulative drug release profile could be plotted. This *in vivo* peptide release profile will enable time point-to-time point comparisons between the *in vitro* and *in vivo* studies. A widely adopted dissolution profile comparison uses similarity factor $f_2$, involving the calculation of difference and similarity factors. These adaptations of the study will therefore, enable the generation of a correct IVIV correlation.
Reference


84. Jain, R.A., et al., *Comparison of various injectable protein-loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices: In-situ-formed implant versus in-situ-


