Controlled release of peptide from biodegradable platforms for cardiovascular applications

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CONTROLLED RELEASE OF PEPTIDE FROM BIODEGRADABLE PLATFORMS FOR CARDIOVASCULAR APPLICATIONS

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fulfilment of the requirement for the degree of Doctor of Philosophy

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Dedication

I would like to dedicate this thesis to my loving parents, god-mother, “fairy” god-mother and my two lovely sisters.

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“Failure is the opportunity to begin again with more intelligence.” Henry Ford (1983-1947)

Table of Contents

Dedication	i
Acknowledgements	ii
Table of Contents	v
List of Abbreviations	x
List of Figures.....	xiv
List of Tables	xx
Abstract.....	1
Chapter 1 Introduction.....	1
1.1 Background	1
1.2 Problem statement.....	4
1.3 Objectives	5
1.4 Scope.....	6
1.5 Overview	7
Chapter 2 Literature Review	9
2.1 Cardiovascular disease and advances in treatment	9
2.1.1 Cardiovascular disease.....	9
2.1.2 Treatment options	12
2.1.3 Challenges and future treatment	17
2.2 Natriuretic peptide	22

2.2.1 Natriuretic peptide (NP) family	22
2.2.2 Therapeutic functions and delivery	28
2.2.3 Designer peptides	32
2.2.4 CD-NP (Cenderitide)	34
2.2.4 Challenges of delivering NP	36
2.3 Biodegradable polymers	37
2.3.1 Biodegradable polymers and biomedical devices	37
2.3.2 Polymer degradation mechanism	40
2.3.3 Factors that affect polymer degradation	41
2.3.4 Poly (ϵ -caprolactone) (PCL)	42
2.3.5 Poly (ethylene glycol) (PEG)	45
2.4 Peptide and protein delivery	48
2.4.1 Prospects and challenges of protein delivery	50
2.4.2 History and developments of protein and peptide delivery	51
2.4.3 Polymeric sustained-delivery systems	54
Chapter 3 Experimental Materials and Method	57
3.1 Materials	57
3.2 Methods	58
3.2.1 Film preparation	58
3.2.2 Surface morphology	59
3.2.3 Confocal laser scanning microscopy (CLSM)	60

3.2.4 In vitro peptide release.....	61
3.2.5 Polymer degradation	64
3.2.6 Cell culture.....	65
Chapter 4 Formulation and film studies.....	70
4.1 Introduction.....	70
4.2 Co-solvent system 1: Ethanol/DCM.....	71
4.2.1 In vitro release.....	72
4.2.2 In vitro degradation.....	73
4.2.3 Surface morphology.....	74
4.2.4 Mathematical analysis.....	77
4.3 Co-solvent system 2: Water/DCM.....	79
4.3.1 In vitro release.....	80
4.3.2 Effects of excipients on in vitro release	81
4.3.3 In vitro degradation.....	83
4.3.4 Surface morphology	84
4.3.5 Peptide distribution	89
4.3.6 Mathematical analysis.....	91
4.4 Discussion	94
Chapter 5 In vitro studies on human cardiac fibroblast cells (HCFs) inhibition	105
5.1 Introduction.....	105
5.2 Effects of CD-NP on HCFs.....	105

5.2.1 Elevation of cGMP production due to CD-NP	106
5.2.2 Effects of CD-NP on HCFs proliferation and viability	107
5.2.3 Effects of multiple dose CD-NP on HCFs proliferation and viability	112
5.2.4 Comparision of single dose and multiple doses CD-NP on HCFs	115
5.3 Effects of released CD-NP on HCFs	116
5.3.1 Review of selected release profiles.....	116
5.3.2 Elevation of cGMP production	118
5.3.3 Effects of released CD-NP on HCFs viability	119
5.2.4 Effects of released CD-NP on HCFs proliferation	121
5.4 Discussion	122
Chapter 6 In vitro studies on human coronary smooth muscle cells (HCaSMCs)	
inhibition.....	128
6.1 Introduction.....	128
6.2 Effects of CD-NP on HCaSMCs.....	129
6.2.1 Elevation of cGMP production in HCaSMCs by CD-NP.....	129
6.2.2 Effects of CD-NP on HCaSMCs proliferation.....	130
6.2.3 Effects of multiple dose CD-NP on HCaSMCs.....	140
6.3 Effects of released CD-NP on HCaSMCs	143
6.2.1 Formulation selection.....	143
6.2.2 Elevation of cGMP production in HCaSMCs by released CD-NP.....	144
6.2.3 Effects of released CD-NP on HCaSMCs proliferation	145
6.2.4 Effects of released CD-NP on HCaSMCs viability	147

6.4 Discussion	148
Chapter 7 Conclusions and Recommendations.....	151
7.1 Conclusions.....	151
7.1.1 Formulation and film studies	151
7.1.2 In vitro human cardiac fibroblast cells (HCFs) study	153
7.1.3 In vitro human coronary smooth muscle cells (HCaSMCs) study	155
7.1.4 Overall conclusion	157
7.2 Recommendations.....	159
7.1.1 Materials perspective	160
7.1.2 Biological and cellular perspective	161
7.1.3 Development of application.....	163
References	164

List of Abbreviations

ANP	= Atrial natriuretic peptide
ACE	= Angiotensin-converting enzyme
AMI	= Acute-myocardial infarction
ANOVA	= Analysis of variance
ASBNP	= Alternative splicing brain natriuretic peptide
BCA assay	= Bicinchoninic acid assay
BMS	= Bare metallic stents
BNP	= Brain natriuretic peptide
BrdU assay	= Bromo-2'-deoxyuridine assay
BSA	= Bovine serum albumin
CD-NP	= Cenderitide
cGMP	= 3', 5' cyclic guanosine monophosphate
CHF	= Congestive heart failure
CLSM	= Confocal laser scanning microscopy
CI	= Cell index
CNP	= C-type natriuretic peptide
CNS	= Central nervous system
CT-1	= Cardiotrophin-1
DCM	= Dichloromethane
DES	= Drug eluting stents
DI	= Deionized

DNA	= Deoxyribonucleic acid
DNP	= Dendroapis natriuretic peptide
DSC	= Differential scanning calorimetry
EC	= Endothelial cells
ECM	= Extracellular matrix
ET-1	= Endothelin-1
ETO	= Ethylene oxide
FDA	= Food and drug administration
FBS	= Fetal bovine serum
FDA	= Food and drug administration
GC	= Guanylate cyclase
GFR	= Glomerular filtration rate
GH	= Growth hormones
GI	= Gastro-intestinal
GTP	= Guanosine triphosphate
H	= high
HAoSMCs	= Human aortic smooth muscle cells
HCaSMCs	= Human coronary smooth muscle cells
HCF	= Human cardiac fibroblast
HEPES	= Hydroxyethyl piperazineethanesulfonic acid
HF	= Heart failure
ISR	= In-stent restenosis
LV	= Intravenous infusion
L	= Low
LE	= Long emulsification

LST = Late stent thrombosis

LV = Left ventricular

M = Medium

MAP = Mean arterial pressure

MI = Myocardial infarction

NEP = Neutral endopeptidase

NGF = Nerve growth factor

NP = Natriuretic peptide

NPR = Natriuretic peptide receptor

NT-pro-BNP = N-terminal pro-hormone brain natriuretic peptide

PBS = Phosphate buffer saline

PBT = Poly (butylenes terephthalate)

PCI = Percutaneous intervention

PCL = Poly (ϵ -caprolactone)

PD = Phosphodiesterase

PDLLA = Poly (DL-lactide)

PEG = Poly (ethylene glycol)

PEO = Poly (ethylene oxide)

PKG = cGMP dependent protein kinase

PLGA = Poly (lactide-co-glycolide)

RCI = Relative cell index

SE = Short emulsification

SEM = Scanning electron microscopy

SMC = Smooth muscle cells

SQ = Subcutaneous infusion

List of Abbreviations

TE = Tissue engineering

TGA = Thermogravimetric analyser

VNP = Vasonatrin natriuretic peptide

List of Figures

Figure 2-1 The cross-section of a) normal vessel, b) plaque accumulated in vessel, c) occluded vessel and d) stented vessel	14
Figure 2-2 The cross-section of a) normal heart, b) infarcted heart, c) re-sectioning of heart and d) heart with patch over.....	16
Figure 2-3 Natriuretic peptide receptors and mechanism	26
Figure 2-4 Structure of CD-NP [13]	36
Figure 2-5 Schematic describing surface and bulk erosion in biodegradable polymers	41
Figure 2-6 Chemical structure of PCL[145]	42
Figure 2-7 Chemical structure of PEG.....	45
Figure 3-1 Calibration curve of CD-NP in buffer solution (pH 7.4) using BCA	62
Figure 3-2 Calibration curve of CD-NP in buffer solution (pH 7.4) after partition in DCM:Buffer (50:50) using BCA.	64
Figure 4-1 Accumulated release profiles of 1% CD-NP loaded films with and without addition of PEG and copolymer PCL(1.5k)-PEG(5k). Accumulated release of a) PCL films with and without addition of PEG and b) PCL films with and without addition of copolymer	73
Figure 4-2 (a) Molecular mass change and (b) mass loss of CD-NP loaded films with and without addition of PEG and copolymer PCL (1.5k)-PEG (5k) over 30days.....	74
Figure 4-3SEM micrograph of 1% CD-NP loaded PCL film, addition of PEG film and addition of copolymer PCL(1.5k)-PEG (5k) on day 0 and after day 30 release.....	76

Figure 4-4 a) Image of polymer-peptide solution stirred under short emulsification (SE) and long emulsification (LE) and b) Accumulated peptide release comparing the effects of SE and LE duration in Pure PCL	80
Figure 4-5 Accumulated CD-NP release from films with and without addition of PEG manufactured under a) short emulsion (SE) and b) long emulsion (LE) conditions	82
Figure 4-6 Accumulated CD-NP release from films with and without addition of copolymer manufactured under a) short emulsion (SE) and b) long emulsion (LE) conditions	83
Figure 4-7 Molar mass change of CD-NP loaded films with and without addition of PEG and copolymer manufactured under a) short emulsion (SE) and b) long emulsion (LE) conditions.	84
Figure 4-8 SEM micrograph of CD-NP loaded PCL film manufactured under a) short emulsion (SE) and b) long emulsion(LE) conditions on day 0 and after day 30 release	86
Figure 4-9 SEM micrograph of CD-NP loaded PCL film with addition of PEG manufactured under a) short emulsion (SE) and b) long emulsion (LE) conditions on day 0 and after day 30 release	87
Figure 4-10 SEM micrograph of CD-NP loaded PCL film with addition of copolymer manufactured under a) short emulsion (SE) and b) long emulsion (LE) conditions on day 0 and after day 30 release	88
Figure 4-11 CLSM image of CD-NP loaded PCL film, 10% addition of PEG film and 10% addition of copolymer PCL-PEG (1.5k-5k) manufactured via short emulsion(SE) and long emulsion(LE) conditions. Each window: 514 Ex, 405 Ex, bright field and Combine	90

Figure 4-12 CLSM image of CD-NP loaded PCL film, 10% addition of PEG film and 10% addition of copolymer PCL (1.5k)-PEG (5k) manufactured via short emulsion (SE) and long emulsion (LE) conditions. Each image represents the combine of 514 Ex, 405 Ex, each section is 6.5 μm with the images from left to right denoting the position of the film from top to bottom.....	91
Figure 4-13 Accumulated peptide release comparing the effects of short emulsion (SE) and long emulsion (LE) duration in PCL formulation, addition of 5% PEG, addition of 10% PEG, addition of 5% copolymer and addition of 10% copolymer formulations..	93
Figure 5-1 Cyclic 3'5' guanosine monophosphate (cGMP) generation in HCF induced by a) 10-fold different and b) 100-fold different CD-NP concentration, * $p < 0.05$ versus control.	107
Figure 5-2 Relative DNA synthesis of different CD-NP concentration in HCF for 24 hours via colormetric bromodeoxyuridine (BrdU), * $p < 0.05$	109
Figure 5-3 Relative DNA synthesis of different CD-NP concentration in HCF for 1,2,3 and 5 days via colormetric bromodeoxyuridine (BrdU), * $p < 0.05$	110
Figure 5-4 Cell Index (CI) measurement of control compared to single dose infusion of different CD-NP concentrations in HCF at 0 day via the RTCA xCELLigence	111
Figure 5-5 Relative DNA synthesis of daily infusion of different CD-NP concentration in HCF for 1,2,3 and 5 days via colormetric bromodeoxyuridine (BrdU), * $p < 0.05$	113
Figure 5-6 Cell Index (CI) measurement of a) daily infusion of different CD-NP concentrations compared to control and b) 0.37 $\mu\text{g/mL}$ daily dose CD-NP in HCF compared to control via the RTCA xCELLigence	114
Figure 5-7 (a) Mode of single dose CD-NP and (b) Mode of daily dose CD-NP	116

Figure 5-8(a) Accumulated peptide release profiles of films L, M and H and (b) Release concentrations of films L, M and H over 30 days	118
Figure 5-9 Cyclic 3'5' guanosine monophosphate (cGMP) generation in HCF induced by 24 hour CD-NP release from films L, M and H, *p < 0.05 versus control.....	119
Figure 5-10 Cell Index (CI) measurement of control compared to daily infusion of CD-NP, films L, M and H in HCF via the RTCA xCELLigence	120
Figure 5-11 Correlation between Relative Cell Index (RCI) (primary y-axis) and peptide concentration (secondary y-axis) of daily infusion of CD-NP, films L, M and H over 5 days (x-axis) in HCF	121
Figure 5-12 Relative DNA synthesis of CD-NP released from films L, M and H (1 day, 2days, 3days and 5days) in HCF via colormetric bromodeoxyuridine (BrdU), *p < 0.05	122
Figure 6-1 Cyclic 3'5' guanosine monophosphate (cGMP) generation in HCaSMCs induced by CD-NP at different concentrations, *p < 0.05 versus control	130
Figure 6-2 Relative DNA synthesis of different CD-NP concentration in HCaSMCs for 24 hours via colormetric bromodeoxyuridine (BrdU), *p < 0.05	131
Figure 6-3 Relative DNA synthesis of different CD-NP concentration in HCaSMCs for 1,3, 5 and 7 days via colormetric bromodeoxyuridine (BrdU), *p < 0.05	132
Figure 6-4 Relative DNA synthesis of different CD-NP concentration in HCaSMCs for 24 hours via colormetric bromodeoxyuridine (BrdU) in a) 4,000 cells/well and b) 12,000 cells/well seeding densities, *p < 0.05	134
Figure 6-5 Relative DNA synthesis of different CD-NP concentration in HCaSMCs for 24 hours via colormetric bromodeoxyuridine (BrdU) under different % FBS conditions, *p < 0.05	134

Figure 6-6 Relative DNA synthesis of different CD-NP concentration in HCaSMCs for 24 hours via colormetric bromodeoxyuridine (BrdU) where SMCs were pre-treated with ET-1, *p <0.05	136
Figure 6-7 Relative DNA synthesis of CD-NP in HCaSMCs for 4,8, 12 and 24 hours via colormetric bromodeoxyuridine (BrdU), *p <0.05	137
Figure 6-8 Relative DNA synthesis of different CD-NP concentration in HCaSMCs for 4 hours via colormetric bromodeoxyuridine(BrdU), *p <0.05	138
Figure 6-9 Relative DNA synthesis of freshly prepared CD-NP solution and CD-NP solution that was incubated in 37 C for 24 hours in HCaSMCs for 4 hours via colormetric bromodeoxyuridine(BrdU), *p <0.05	139
Figure 6-10 Relative DNA synthesis of single versus multiple doses of CD-NP in HCaSMCs via colormetric bromodeoxyuridine(BrdU), *p <0.05.....	140
Figure 6-11 Cell Index (CI) measurement of control compared to single dose infusion of different CD-NP concentrations in HCaSMCs at 0 day via the RTCA xCELLigence	142
Figure 6-12 Cell Index (CI) measurement of a) daily infusion of different CD-NP concentrations compared to control in HCaSMCs compared to control via the RTCA xCELLigence	143
Figure 6-13 Cyclic 3'5' guanosine monophosphate (cGMP) generation in HCaSMCs induced by 24 hour CD-NP release from PCL films, 10% PEG and 10% copolymer, *p < 0.05 versus control.....	145
Figure 6-14 Relative DNA synthesis of CD-NP released from different CD-NP loaded formulation (released in 1 day, 3days and 7days) in HCaSMCs via colormetric bromodeoxyuridine (BrdU), *p <0.05	146

Figure 6-15 Cell Index (CI) measurement of control compared to CD-NP released from different CD-NP loaded formulation in HCaSMCs via the RTCA xCELLigence	147
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List of Tables

Table 2-1 Summary on Natriuretic peptide origin and major functions [65, 68, 73-79]	24
Table 3-1 Table of materials and reagent	57
Table 3-2 Film formulation and peptide loading	58
Table 3-3 Excitation and emission wavelengths of molecules	61
Table 4-1 Similarity and different factor comparing formulations in ethanol/DCM system	79
Table 4-2 Different and similar factor comparing the release profiles between short emulsification and long emulsification of PCL and formulations with addition of PEG and copolymer in water/DCM system	94
Table 5-1 Formulations to be tested for HCF studies	117
Table 6-1 Formulations to be tested for HCaSMCs studies	144

Abstract

Cenderitide, also known as CD-NP, is a designer peptide developed by Mayo Clinic for treating cardiovascular pathologies. CD-NP is synthesized by combining native mammalian c-type natriuretic peptide (CNP) and a C-terminus isolated from the dendroaspis natriuretic peptide (DNP) of the venom of green mamba. Currently, the clinical means of administering CD-NP is via intravenous or subcutaneous injection. However, such administration is low in patient compliance and results in high wastage.

This thesis presents the development of a controlled release system for the administration of CD-NP over a sustained period of time. Due to difficulty in solubilizing hydrophilic CD-NP, two co-solvent systems were explored for the encapsulation of CD-NP in polymeric matrix. In the first co-solvent system consisting of ethanol and dichloromethane (DCM), sustained release of CD-NP from PCL-based formulation was achieved for 30 days. The release rates of CD-NP were further manipulated through the addition of polyethylene glycol (PEG) and copolymers of poly (ϵ -caprolactone) with PEG. The addition of these excipients resulted in increase in the initial CD-NP release. A detailed investigation on the surface morphology of films, degradation characteristics and kinetics of release was carried out and the co-leaching of CD-NP with excipient was singled out as the key factor for the increase in the initial release. In the second co-solvent system consisting of water and DCM, two emulsification conditions were studied due to the immiscibility of the two solvents. The results demonstrate that different emulsification conditions dramatically affected

the initial release of peptide, where a difference of 5-fold was observed. This observation was attributed to difference in peptide aggregation and degree of porous formation under the two emulsification conditions. Subsequently, the combined effects of emulsification condition and excipient addition were investigated. The addition of PEG and copolymer reduced the effects of emulsification on peptide release due to co-localization of excipients with peptide, which redistributed CD-NP throughout the matrix.

In literature, CD-NP had been reported to possess anti-fibrotic and anti-proliferative properties. However, these studies lack systematic understanding on the extent of CD-NP's actions on cardiac cells in vitro. In this work, the anti-fibrotic capability of CD-NP was explored through the study of CD-NP's interactions with the human cardiac fibroblast (HCF) cells. First, we studied the effects of CD-NP on HCF and elucidated the extent of CD-NP inhibition on HCF. Single dose CD-NP exhibited inhibition of hyperplasia HCF for up to 24 hours but was ineffective on hypertrophic HCF. Multiple doses CD-NP sustained the inhibition of hyperplasia HCF for 5 days for the highest concentration. Moreover, multiple doses of CD-NP at highest concentration exhibit inhibition of hypertrophic HCF by the third dose. We also studied the anti-proliferative capability of CD-NP by incubating CD-NP with the human coronary smooth muscle cells (HCaSMCs). We found that the inhibitory effects of CD-NP on HCaSMCs were limited compared to HFC.

After elucidating the effects of CD-NP on the two cardiac cell types, we moved to understand the effects of CD-NP released from the controlled release systems on HCF and HCaSMCs. The bioactivity of released peptide was verified from the elevated

production of cyclic guanosine monophosphate (cGMP) in both HCF and HCaSMCs. To understand the effects of continuous release CD-NP, selected films releasing CD-NP were incubated with the HCF. The sustained delivery of CD-NP from formulations manufactured from Water/DCM co-solvent system showed superior suppressive actions on HCF compared to daily infusion of CD-NP. Furthermore, we investigated the effects of excipient addition on encapsulated CD-NP. The addition of excipients did not result in loss of bioactivity in the released CD-NP, which signified that the addition of excipients could be used as modifier or stabilizer in peptide release profiles. The results suggest that development of CD-NP releasing films or devices could be used to inhibit fibrosis and reduce cardiac remodeling in future studies.

Chapter 1 Introduction

1.1 Background

Cardiovascular disease is the leading cause of death in the world. It accounts for 30% of the death in the world and is a huge financial burden to the patients and their family. Amongst the cardiovascular diseases, the occlusion of the artery due to atherosclerotic rupture is the most common cause of mortality. In fact, 70% of fatalities are a resultant of artery occlusion. The occlusion results in reduction of blood, nutrients and oxygen transportation to the myocardium. Currently, the most common surgical intervention is balloon angioplasty accompanied by the implantation of a bare metal stent (BMS) or a drug eluting stent (DES). The insertion of BMS leads to unacceptable restenosis (re-blockage of the stented vessel) whereas the use of non-selective anti-proliferative therapeutic agents in the DES leads to delayed endothelialization and sometimes results in fatal late-stage thrombosis (LST).

If intervention is not carried out in time, excessive necrosis occurs in the myocardium and left ventricular (LV) remodeling is triggered. The heart attempts to preserve cardiac output by dilating the ventricular chamber, which leads to thinning of ventricular wall and modification of ventricular sphericity [1-6]. These changes result in the loss of contractile function, decreased output and ultimately congestive heart failure (CHF) [3, 5-7]. Currently, ventricular surgeries are the most widely used procedures in hospitals. Unfortunately, these procedures only serve to temporarily

reduce the chamber volume but do not provide any therapeutic assistance in preventing fibrotic spread or restoration of the contractile functions.

In cardiovascular treatment of artery occlusion and LV remodeling, the lack of the appropriate therapeutic agent and delivery vehicle are major problems. For instance, the anti-proliferative drugs used in DES inhibited the neointima proliferation, reducing restenosis; however, the non-selectivity of their action results in delayed endothelialisation of the vessel [8-10]. Although cardiovascular therapeutics like angiotensin-converting enzyme (ACE) inhibitors, beta-blockers and aldosterone antagonist [11, 12] relieve some cardiovascular symptoms, their side effects make them unsatisfactory. Similarly, the only intervention for ventricular remodeling is surgical procedure and it involves mechanical constraints and management of dilation alone; these do not adequately halt fibrosis progression and are invasive.

Novel Therapeutic Approaches to Management of post-MI Conditions

One promising therapeutic approach is using natriuretic peptides as therapeutic agents because they show activity following an MI. Their role in ventricular remodeling has been extensively studied in recent years [13-16]. They play roles in the relaxation of constricted vessels and the regulation of blood pressure. However, each peptide has beneficial and detrimental effects. For example, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) possesses natriuretic properties but they are hypotensive. On the other hand, the c-type natriuretic peptide (CNP) has vasodilating effects but lacks the desired renal effects. To achieve the best of the different peptides while minimizing the detrimental effects, the development of novel designer peptides have emerged. One such chimeric peptide, CD-NP was developed by Mayo Clinic. It

achieved positive results in preliminary human studies and has garnered much attention. CD-NP is formed by fusing the C-type natriuretic peptide (CNP) with the C-terminus of the dendroapais natriuretic peptide (DNP) isolated from a green mamba. Preliminary studies suggest that CD-NP possesses potent anti-fibrotic and anti-proliferation properties. Such properties make CD-NP extremely desirable in attenuating cardiovascular pathologies associated with smooth muscle proliferation (following stenting) and collagen accumulation (post-MI).

However, the clinical use of NPs had been largely hindered by its short half-life, delicate nature and the absence of suitable delivery and administration routes. Currently, the means of delivering NPs have been via intravenous or subcutaneous infusion. These routes of administering CD-NP were effective in elevating plasma cGMP, lowering blood pressure and unloading the heart. However, IV infusions have to be done in a hospital setting, continuously for several days or weeks following a MI; the sub-cutaneous administration is given via an implanted pump, which requires hospital visits for implantation and removal, and it is also uncomfortable to the patient. Moreover, such systematic delivery is low in efficacy as the pathology is not targeted locally.

CD-NP is a relatively new therapeutic agent that holds a lot of potential to circumvent cardiovascular pathological conditions that can benefit from novel sustained delivery approaches. To the author's knowledge there has been no research published on the understanding of controlled release of CD-NP from polymeric implants/ devices. With the need to bridge the gap in knowledge, the basis of the thesis is formed, where the author would investigate and attempt to understand the release of CD-NP from

polymer matrices. In this work, we explored systems for the encapsulation and the delivery of CD-NP from polymeric platforms. Also, we attempted to establish a systematic understanding on the effects of CD-NP on cardiac cells, in particular the effects of dose and sustained release.

1.2 Problem statement

CD-NP has a short biological half life; hence continuous supply is needed to maintain the therapeutic dose. It appears that developing an implantable device encapsulating and releasing CD-NP to the desired site is appealing. Furthermore, the release of CD-NP in a continuous and sustained manner within the therapeutic range is ideal. *To the author's knowledge, there has been no research published on the development and understanding of controlled release CD-NP from polymeric implants/ devices.*

CD-NP is regarded as a potent therapeutic agent, yet there is no systematic study on the extent of their effectiveness. CD-NP had been reported to possess anti-fibrotic and anti-proliferative actions on human cardiac fibroblast cells (HCFs) and human coronary smooth muscle cells (HCaSMCs) respectively. However, these studies only investigated on single end-point and the extent of their inhibition had not been reported. *There is a lack of knowledge on the extent and effective anti-proliferation and anti-fibrotic actions of CD-NP.*

The pathological mechanism of cardiac diseases had been intensively studied, yet the management of heart failure remains poor due to the lack of appropriate therapeutic

agents and effective administration routes. The continuous delivery of CD-NP via intravenous infusion (IV) and subcutaneous infusion (SQ) have yielded encouraging cardiac responses. However, these means of delivery are non-localized, wasteful and low in patient compliance. *There have been no reports on the effects of sustained release CD-NP from polymeric platforms on the inhibition of HCaSMCs or HCFs.*

1.3 Objectives

There are three primary goals in this work. The first goal is to study and establish CD-NP release from polymer and its blends over a sustained period of time. The second goal is to understand the extent (dose dependence) of CD-NP's biological actions on cardiac cells—HCFs and HCaSMCs. The third goal is to elucidate the effects of controlled release CD-NP on HCFs and HCaSMCs.

To achieve these objectives, the work is divided into the following:

The first part focuses on developing polymer matrices for the release of CD-NP. Two different co-solvent systems were used to develop the matrices for the entrapment of CD-NP. To obtain different release trends, the effects of excipients addition were explored. To understand the influences of these parameters on the release mechanism, the morphology, polymer degradation and peptide distribution were investigated. To understand the impact of excipient addition and emulsification duration, formulations were cross-compared and statistical analysis was carried out.

The second part attempts to study the effects of CD-NP on HCF. Firstly, the effects of CD-NP on cardiotrophin-1 (CT-1) induced proliferation of HCFs were studied. The cell viability, DNA synthesis and its ability to elicit 3', 5' cyclic Guanosine Monophosphate (cGMP) response in HCF were investigated. The proliferation activities of HCF, dose and duration of CD-NP interaction with HCF were investigated to understand the extent of HCF inhibition. Next, the effects of selected release formulations on the proliferation of HCF were investigated. Also, the cGMP assay was used to further verify the retention of bioactivity of release CD-NP. Finally, the effects of pure CD-NP on HCF were compared with the effects of released CD-NP.

The third part investigates the effects of CD-NP and encapsulated CD-NP on HCaSMCs. Firstly, the ability of CD-NP to elicit cGMP response and inhibit HCaSMCs' proliferation was investigated. After elucidating the extent of CD-NP on HCaSMCs inhibition, CD-NP released from different formulations with and without excipient was evaluated for their retention of bioactivity.

1.4 Scope

This work studies the encapsulation of chimeric peptide, CD-NP in poly (ϵ -caprolactone) (PCL) solvent cast films. Two types of excipients were explored and they are poly (ethylene glycol) (PEG) (2k) and copolymer PCL (5k)-PEG (1.5k). The release study was carried out over 30 days by immersing cut films in PBS and incubating them in the 37 °C incubator. The degradation study was carried out separately in a similar fashion to the release study. The degradation study consisted of

mass loss, molecular mass change and surface morphology investigation. Further verification of the peptide distribution was studied using the confocal laser scanning microscopy (CLSM).

For the in vitro cell studies, the human cardiac fibroblast (HCF) and the human coronary artery smooth muscle cells (HCaSMCs) were investigated. To assess the cellular activities, the cGMP assay was used to observe the intracellular cGMP level. For the cell viability and proliferation, two methods were employed. An ELISA assay, 5'-bromo-2'-deoxyuridine (BrdU), was used to determine the amount of DNA synthesis, which gives an indication of cell proliferation. And the xCELLigence system is based on cellular activity-induced impedance, where it monitors real-time cell viability and proliferation.

1.5 Overview

This thesis is organized into seven chapters. In this foremost chapter, the background, rationale and scope of this work were highlighted. Chapter 2 presents a review of reported literature in four sections: (1) cardiovascular disease, (2) natriuretic peptide, (3) biodegradable polymers, and (4) peptide delivery. The review focuses on giving insights on the problems and the advances in the field. The review also highlights some unresolved issues, which puts our work in perspective and sets the directions of this project. Chapter 3 describes the materials and methods utilized in this work. The results and discussion of this work is presented in chapters 4, 5 and 6. Chapter 4 focuses on the encapsulation and the release of CD-NP from polymer PCL platforms,

where we examine the influence of different co-solvent systems and the effects of addition of excipients on film properties and the release of CD-NP. In chapter 5, we seek to understand the inhibitory effects of CD-NP on HCF, which is imperative for the selection of formulations for further investigations. In the latter part of the chapter, we selected low, moderate and high initial release profiles to understand which profiles showed better inhibition of HCF. In chapter 6, we examined the effects of CD-NP on HCaSMCs. Upon understanding the inhibitory behavior, we selected different formulations to understand if the addition of excipient enhanced or declined the inhibitory effects of released CD-NP. In our final chapter, we draw conclusions to the work investigated and propose future recommendations.

Chapter 2 Literature Review

2.1 Cardiovascular disease and advances in treatment

In section 2.1, the review on cardiovascular disease and advances in treatment is subsectioned into the section 2.1.1, section 2.1.2 and section 2.1.3, where the pathology, the current treatment options and the challenges are reviewed respectively.

2.1.1 Cardiovascular disease

Cardiovascular disease is the number one cause of death in the world. In 2008, world health organization (WHO) estimated that 17.3 million people died from cardiovascular disease, accounting for 30% of the death in the world [17]. Due to the growth in the aging population, cardiovascular disease is likely to continue to be the main cause of death in the future.

The clinical, social and financial burden of coronary syndromes is severe. Based on the Framingham Heart study [18], one in five adult men and women risk developing congestive heart failure (CHF) in their lifetime. Despite the advances in medical technologies and improvements in healthcare management, the battle against CHF is an uphill fight [11, 12, 19, 20]. In a long term study done by Levy D et al [21], one in three patients diagnosed with CHF does not survive more than a year. And almost half of the remaining patients died within 5 years.

Heart failure is the terminal of most cardiovascular diseases. Cardiovascular diseases can be broadly defined as the pathology of the heart and vessels; and some of the conditions include hypertension, cardiomyopathy, coronary atherosclerosis and congenital heart malfunctions [6, 22]. In this part of the literature review, we focus on coronary atherosclerosis (leading to acute myocardial infarction (AMI)) and LV remodeling (post-AMI).

Coronary atherosclerosis

Atherosclerotic rupture in occluded vessel is the most common cause of AMI. In fact, 70% of hospitalizations are a resultant of artery occlusion [23]. In the following, we describe the progression of AMI from atherosclerosis. The progression of atherosclerosis could be simplified into the following phases:

In the initial phase, plaques start to develop on the arterial wall. In this phase, the cross-section of the lumen is largely conserved though the plaque region may undergo outward remodeling to compensate [24, 25].

In the progressive phase, plaques accumulation over time causes the lumen to be occluded [26]. In this phase, the constricted vessel may cause disruption of blood flow and blood pressure.

In the final phase, plaques fissure and rupture occurs causing the lumen to be totally occluded. The blood flow, nutrients and oxygen supply to the myocardium is entirely cut off; cardiac cells undergo ischemia and hypoxia. If the occlusion persists beyond 30 minutes, the myocardium undergoes irreversible damage and AMI is triggered [25].

LV remodeling

Post AMI, the heart undergoes a series of changes in an attempt to compensate the damage. The series of compensatory mechanism is known as LV remodeling and the progression could be described in the following phases:

In the initial phase, small regions of myocardium are damaged and the heart may experience decline in pumping capacity. In this phase, the adaptive compensatory mechanism is activated where the uninjured regions of the heart works harder to preserve the normal functions. On the cellular level, cardiac cells undergo hypertrophy in accordance to Starling law, hence increasing the cardiac output to compensate the damage [27].

In the progressive phase, the heart continues to undergo increase in cardiac load, resulting in decline in pumping capacity. As the heart attempts to maintain the pressure difference, greater tension is developed within the cardiac muscle, resulting in dilation of the heart in accordance with the Laplace law [28]. In this phase, the adaptive compensatory mechanism progresses to maladaptive mechanism, where the heart starts to undergo chamber dilation and changes in its shape.

In the final phase, the heart undergoes functional deterioration along with grossly abnormal features. In this phase, the heart has changed its ellipsoid shape to spherical shape. Moreover, the heart undergoes severe thinning of its wall and massive dilation. If the process continues, all contractile functions diminish and the heart is impaired beyond reversible repair.

2.1.2 Treatment options

In this section, the treatment options for vessel occlusion and cardiac remodeling is described in sections 2.1.2.1 and 2.1.2.2 respectively.

2.1.2.1 Treatment for vessel occlusion

Since thrombotic occlusion within the vessel is the main cause of AMI, the current treatment strategies had been targeted at restoring the blood circulation of the coronary system. Currently, reperfusion regimens and surgical intervention are the common strategies employed by the cardiologists.

Reperfusion therapy

Reperfusion with thrombolysis agents is the most immediate pharmacological means to break down the blood clots and restore circulation [26]. At present, common thrombolysis agent used includes streptokinase and alteplase via intravenous injection [29-31]. Both agents have been generally regarded as effective because they showed reduction in mortality [30], although, some have regarded front-loaded alteplase as the gold standard due to better patency outcome [31]. Despite the ease of application of reperfusion therapy, the stringent effective window period and serious side effects have limited its usage, which would be discussed in greater details later.

Surgical Intervention

Percutaneous coronary intervention (PCI), also known as balloon angioplasty is a commonly used procedure due to broader effective window period and higher

effectiveness compared to thrombolytic perfusion. PCI procedure involves the delivery of a balloon tipped tube attached at the end of a catheter from the femoral artery to the injured segment of the coronary artery. When the balloon arrives at the site of occlusion, it is inflated, crushing the plaques into the walls of the artery and allowing the blood to flow freely. Subsequently, the balloon is deflated and the catheter is withdrawn from the patient's body.

Cardiovascular stents

Over the years, PCI had advanced dramatically in terms of procedure, medication and most importantly the device itself. Today, the procedure is normally accompanied by an expandable metal mesh, known as the bare metallic stent (BMS). The stents serves to keep the vessel patent post PCI by overcoming the negative modeling experienced by the vessel [32, 33]. Cardiovascular stents had successfully aided the PCI procedure in terms of overcoming stenosis and elastic recoil. However, stents implantation can induce acute local inflammation which gives raise to other issues including restenosis (re-narrowing of artery) and thrombosis (formation of thrombus). Figure 2.1 is a pictorial description of vessel occlusion and stents usage.

An important enhancement to the BMS is the development of Drug-Eluting Stents (DES). Apart from serving its role to keep the vessel patent, DES releases therapeutic agents to prevent the overgrowth of scar tissues, hence eradicating the problem of restenosis. In fact, the first two DES stents approved by the Food and Drug Administration (FDA) and widely used in the market, are the Paclitaxel-eluting stent (TAXUS®) and sirolimus-eluting stent (Cypher®)[8, 10, 34]. These DESs have been able to significantly reduce the incidence of restenosis in stented segments. However,

the usage of these anti-proliferative drugs causes delay in the endothelial healing, resulting in late stent thrombosis (LST) which is often fatal [9, 35-37].

Figure 2-1 The cross-section of a) normal vessel, b) plaque accumulated in vessel, c) occluded vessel and d) stented vessel

Others

The bypass surgery is considered if the reperfusion therapy and percutaneous intervention fails to achieve the desired outcome. The bypass surgery is a procedure where arteries or veins from another part of the patient's body are removed and grafted onto the coronary artery to circumvent the atherosclerotic artery, hence restoring the blood flow.

2.1.2.2 Treatment for cardiac remodeling

Failure to arrest progression and spread of infarcted ventricles is detrimental and potentially fatal. Current treatment strategies to counter cardiac remodelling could be broadly categorized into “replacement”, “salvage” and “restorative” strategies.

Heart transplant

The “replacement” strategy is the best treatment option. In fact, heart transplant is the gold standard treatment particularly for end-stage heart failure patients. Unfortunately, due to the shortage of available donor hearts, only limited number of patients could benefit from this treatment.

Ventricular surgical procedures

The next best strategy is the “salvage” strategy which involves surgical procedures. For almost 3 decades, the ventriculoplasty surgical procedures had been extensively practiced to reduce LV cavity volume, restore ventricular elliptical shape and reduce ventricle wall tension [38]. Two of the classic surgical procedures include the Dor and Batista procedure, where ventricular size is reduced via stitching or removal of a portion of tissue.

The Batista procedure also known as the partial left ventriculectomy is a procedure that involves the partial removal of LV muscle [39]. The procedure had yield favorable immediate and short-term effects, where successful reduction in wall tension and increase in ejection fractions were observed [40]. However, due a substantial number of late failures, many hospitals have eventually abandoned the procedure [38, 41].

Today, the Dor procedure is the most performed surgical procedure [28, 38, 42]. It is also known as the endoventricular circular patch plasty due to the usage of a circular patch in the procedure. The procedure involves the removal of aneurysm, followed by patching the area with a circular patch [43, 44]. To maintain sufficient LV volume, a

balloon or spacer of known volume (60 to 70cc/m²) is placed in the LV cavity throughout the entire procedure. Figure 2.2 is a pictorial description of LV remodeling and the Dor procedures.

Figure 2-2 The cross-section of a) normal heart, b) infarcted heart, c) re-sectioning of heart and d) heart with patch over

Regenerative therapy: Cell therapy and tissue engineering

Tissue engineering is a “restorative strategy” that has received a lot of attention. The appearance of the ‘Vacanti mouse’ (rodent with human ear-shaped cartilage grown on its back) in the late 1990s further garnered worldwide eyeballs. The shortage of donor heart and lack of functional benefits of surgical procedure is one of the biggest motivations behind tissue engineering solutions for cardiovascular pathologies. In a nutshell, the long-term goal of tissue engineering is to produce an entire functional organ to replace failed organs using the patient’s own cells (if possible), hence reducing complications associated with allografts and axografts.

Creating an entirely functional in vitro organ is very complex. For instance, the synchrony of mechanical and electrical functions and the sustainability of the heart needs to be considered. Hence, much research had been focused on developing parts of the heart instead of the entire organ. In fact, several research groups had been actively developing tissue engineered (TE) patches [45-49]. The idea of these TE patches is to promote cardiac regeneration via seeding stem-cells derived cells or primary cardiomyocytes into scaffolds. Ideally, the regenerated cells would couple electrically and mechanically to the host myocardium, restoring cardiac functions. However, high regulatory barriers have hindered immediate clinical translation of these TE patches.

Ventricular devices

In the recent years, minimally invasive surgeries to deliver novel devices have been developed. Two such devices are the “heart parachute” (CardioKinetics, Menlo Park) [50, 51] and the “heart jacket” (Acorn Corcop cardiac support device) [52, 53]. The concept of the “heart parachute” is to partition the enlarged LV into static and dynamic chambers by sealing off the static chamber from the circulation; the LV wall stress is decreased hence reducing the stress of LV dilation. On the other hand, the principle of the “heart jacket” is to restrain the LV, preventing progression of further remodeling and LV dilation. Studies on these devices had shown improvement in the cardiovascular hemodynamic. In fact the “heart jacket” had received CE mark approval.

2.1.3 Challenges and future treatment

In this section, the challenges pertaining to the treatment options for vessel occlusion and cardiac remodeling is described in sections 2.1.3.1 and 2.1.3.2 respectively.

2.1.3.1 Evaluation of cardiovascular artery disease treatment options

From the review presented in section 2.1.2.1, 3 primary treatment options to counter vessel occlusion were presented. In this section, we discuss the limitation and future of reperfusion therapy, PCI and bypass surgery.

Limitations of reperfusion therapy

The reperfusion therapy is the treatment option with the most stringent limitations. The usage of reperfusion therapy is limited to first-time heart attack patients due to the development of immunity against the bacterial-based thrombolytic agents. Moreover, prompt reperfusion is required within limited effective window period of 4 hours post-acute arterial occlusion. And beyond 12 hours post attack the risk outweighs the benefits [29, 54]. Furthermore, patients risk incidence of intracranial bleeding after reperfusion therapy.

Despite the limitations mentioned, the reperfusion therapy proves to be an effective and immediate strategy in easing the conditions for patients who are eligible. However, for patients with attack history or have no access to immediate treatment, the reperfusion therapy is not a viable treatment option.

Limitations of PCI and DES

For patients with coronary artery blockage, the bare metallic stents (BMS) and drug-eluting stents (DES) serves as mechanical means to patent the vessel. DES gained preference over BMS due to its ability to elute anti-proliferative drugs, hence restraining the neointima proliferation.

However, the gratifying outcomes of DES is often halted by the non-selectivity of the drug for SMCs and endothelial cells (ECs), resulting in delayed endothelialisation of the vessel [8-10]. The lack of adequate healing gives arise to late-stage thrombosis (LST) and in-stent restenosis (ISR) post PCI, which prolongs the use of dual anti-platelet therapy in stented patients, and in some cases, exacerbating to fatal LST.

Future of DES

The success of DES in terms of patient mobility and mortality has been undeniable. However, to tackle the other problems that arose due to the use DES, we could consider tackling the problem from the materials and the therapeutic agent perspectives.

From the materials perspective, many groups had been working on fully biodegradable stents for a while. Although coated DESs had been extensively used, the usage of metallic stents often brings about the issues of non-biodegradability, inflammation due to the exposure of struts which often leads to platelet aggregation and hypersensitivity. The development of a fully biodegradable stent could eradicate the above mentioned problems, hence making this option extremely attractive.

The other major problem lies in the therapeutic agents used in DES. The DES had been preferred over BMS due to its ability of eluting anti-proliferative drugs to inhibit the growth and migration of SMCs. However, the drugs used in DES are non-selective. They delay endothelial healing which leads to late-stage thrombosis (LST) and in-stent restenosis (ISR). Hence, the usage of *an ideal therapeutics that selectively inhibits SMCs whilst not affecting the growth of endothelial cells* is desired.

Limitations of bypass surgery

For the past 2 decades, the risk and benefits of bypass surgery had been extensive debated [55] due to inconsistent and contradictory results. Moreover, surgical trauma is one of the main concerns. Complications such as cognitive impairment had been reported, where 51% of patients experienced significant brain damage post-surgery [56]. Furthermore, the patients risk stroke or even death during the surgical procedures. However, the bypass surgery may be necessary for patients with multi-vessel occlusion where the usage of PCI and DES are not viable options.

2.1.3.2 Evaluation of LV remodeling treatment options

From the review presented in section 2.1.2.2, the treatment options to counter LV remodeling were explored. In this section, we discuss the limitations and future of these treatment options in view of the persistent shortage of donor hearts.

Limitations of ventricular surgeries

The application of ventricular angioplasty surgeries, particularly the Dor surgical procedures had been extensively used in hospital to reduce chamber volume. However,

the surgical trauma, large incisions and slow recovery associated with open-chest surgeries had made these surgeries extremely unappealing [3]. Moreover, the Dor procedure involves the usage of Dacron patches, which are not biodegradable.

Limitation of technology

Despite the hype and enthusiasm for research in the cardiac tissue engineering field over the last decade, the technology is not ready for immediate clinical translations. There are major barriers that hinder the clinical potential of this technology. Firstly, the lack of vascularization is the main cause of failure in tissue survival [57-59]. Due to the limits of oxygen diffusion distance (approximately 200 μ m), cells were found proliferating at the edges of the constructs while the cells in the central regions were largely necrotic. Secondly, much of the success seen in the field had been carried out using neonatal rat cardiomyocytes, which are not clinically viable [59]. Thirdly, there are many ethical and practicality concerns surrounding cell source and cell transfer efficiency.

Future of devices

Open-chest ventricular surgical procedures are coupled to procedure-related mortality, hence the quest for minimally invasive procedure is constantly pursued. Moreover, the deployment of minimally invasive methods can significantly reduced infection risk and speed up the recovery time [38, 50-53].

Earlier, we discussed two potential devices—the “heart parachute” (CardioKinetics, Menlo Park) and the “heart jacket” (Acorn Corcop cardiac support device). Methods to

deploy these devices via minimally invasive surgeries is available, hence they may be potential post cardiac remodeling procedures in the near future.

However, affixing the “heart jacket” to the infarcted region only serves to temporarily reduce the chamber volume via mechanical means. And affixing the “heart” parachute only serves to temporarily partition the chambers via physical means. These devices provides either mechanical or physical support but *fails to provide any functional benefits in preventing fibrotic spread or restoration of contractile functions*. Equipping these devices with *functional benefits* is likely to amplify the benefits and should be considered in the near future.

2.2 Natriuretic peptide

Section 2.2 is sub-sectioned into five sections. In section 2.2.1, the discovery, structure and receptors of natriuretic peptide (NPs) is reviewed. Section 2.2.2 reviews the therapeutic functions of NPs, section 2.2.3 reviews the existing designer peptides and section 2.2.4 reviews the NP of interest—CD-NP. Finally in section 2.2.5, we review the options and challenges of NPs delivery.

2.2.1 Natriuretic peptide (NP) family

Introduction of NP

Natriuretic peptides (NP) are a group of hormones with a distinct 17 amino acid disulphide ring. To date, there are four main types of natriuretic peptides: atrial

natriuretic peptide (ANP), brain natriuretic peptide (BNP), c-type natriuretic peptide (CNP) and dendoaspis natriuretic peptide (DNP). The first three mentioned belongs to the mammalian NP family whilst the latter was isolated from the venom of a green mamba. The NPs in the NP family are similar in structure but are genetically diverse with distinctive functions in the cardiovascular, renal and endocrine homeostasis [60-62]. The NP family yields its name due to the discovery of potent natriuretic properties in ANP, however not all NPs in the family possess the function to regulate sodium excretion [63]. In the past decade, the establishment and understanding of the physiological and functional roles of NPs had been intensively investigated [64-71].

Discovery and synthesis

In 1981, de Bold and his co-workers discovered that the injection of atrial myocardial extracts in rats induced natriuretic responses. This discovery eventually led to the identification and isolation of an atrial hormone which is now known as ANP. Following this discovery, Japanese investigators Sudoh and co-workers isolated BNP and CNP from the porcine brain in 1988 and 1990 respectively. And DNP was isolated from the venom of a green mamba by Schweitz et al in 1992.

As its name suggests, the major site of ANP synthesis is in the cardiac atria. Although, BNP and CNP were isolated from the brain tissues of porcine, BNP is mainly produced in the ventricle while CNP is dominantly produced in the central nervous system (CNS) and vascular endothelial cells (ECs).

The following table 2.1, is a summary on the origins and structural information [72] of the mammalian NP.

Table 2-1 Summary on Natriuretic peptide origin and major functions [65, 68, 73-79]

	Native Natriuretic Peptides		
	ANP	BNP	CNP
Origin	Mainly produced in the atrial myocardium.	Mainly produced in the ventricle.	Produced in endothelial cells.
Structure
Stimulus for secretion	1. Increase in atrial stretch and pressure 2. Presence of endothelin and angiotensin II	1. Increase in atrial stretch and pressure 2. Presence of endothelin and angiotensin II	Presence of <u>cytokines</u> : transforming growth factor- β , tumor necrosis factor, IL-1 β , IL- α , fibroblast growth factors and lipopolysaccharide
Half-life (in plasma)	3.2 minutes	22 minutes	2.6 minutes
Interaction of NP with high affinity NP receptors(NPR-)	<u>NPR-A</u> which is abundant in large blood vessels binds to both ANP and BNP.		<u>NPR-B</u> which predominates in the brain binds to CNP.

NP receptors

There are three known natriuretic peptide receptors (NPR)—known as NPR-A, NPR-B and NPR-C [73]. Due to possession of guanylate cyclase (GC) catalytic domain and activity, NPR-A and NPR-B are also referred as GC-A and GC-B respectively.

cGMP elevation

ANP, BNP and DNP displays high affinity to NPR-A and possessed the ability to activate NPR-A [68, 74, 75]. CNP is unable to activate NPR-A due to the lack of C-terminus. Only CNP possesses high affinity to NPR-B and the ability to activate GC-B [15, 76-78]. Upon activation of NPR by binding with respective NPs, the intracellular concentration of cyclic guanosine monophosphate (cGMP) is elevated. The elevation of cGMP leads to the activation of cGMP-dependent protein kinase (PKG) and decrease of cytosolic free calcium ions. The activation of PKG and phosphodiesterase (PD) mediates physiological functions such as vaso-relaxation, cardiac unloading and homeostasis. Finally, cGMP-dependent signaling is terminated by cGMP-dependent PD. Physiologically, the elevation of cGMP had been reported to mediate the inhibition of renin and aldosterone, vasorelaxant, anti-fibrotic and anti-hypertrophic actions.

Clearance

There are 2 routes which NPs are cleared from the circulations—clearance receptor and enzymatic degradation. NPs could be cleared via NPR-C which is commonly known as the clearance receptor. Unlike NPR-A and NPR-B, NPR-C lacks guanylate cyclase (GC) catalytic. NPR-C is the most abundant receptor and it is widely

distributed. NPs are cleared from the blood via enzymatic degradation by neutral endopeptidase (NEP). Since CNP has higher affinity to NPR-C, some groups have also suggested that NPR-C played roles in mediation of physiological actions of some hormones and regulation of vascular tones. Figure 2.3 is a summary of NP interaction with respective NPR.

Figure 2-3 Natriuretic peptide receptors and mechanism

Circulation levels in the body

In physiological state, the production of ANP in the ventricular is low. However, in pathological conditions including ventricular hypertrophy, pressure overload and presence of neuro-humoral factors (such as endothelin-1) stimulate the release of ANP. In healthy adults, the plasma level of ANP is around 10 fmol/mL. In patients with CHF, the level could be 10 to 30-folds higher [79].

Similarly, BNP level in ventricle is typically low and the secretion of BNP is depended on the venous volume and blood pressure. Additionally, ischemia and hypoxia also induces the production of endogenous BNP. In healthy adults, the plasma level of BNP is around 1 fmol/mL. In patients with CHF, the level could be 200 to 300-folds higher [79]. The diverse range in plasma BNP is a good diagnostic tool to differential healthy individuals and individuals at risk of HF.

Like ANP and BNP, the plasma level of CNP increases with the clinical severity of patients. In normal adults, the plasma level of CNP is around 8 pg/mL [80]. However, the level of CNP in healthy adults and CHF patients is reported to have a difference of 2 to 3-fold.

In patients with CHF, BNP plasma level elevation is more significant compared to ANP and CNP, hence it have been regarded as the most useful indicators in clinical practice [81]. BNP is actually produced from pro-hormones (pro-BNP). When the pro-BNP is secreted, it breaks itself into the biologically active BNP and N-terminal pro-hormone BNP (NT-pro-BNP) fragment. Currently, both BNP and NT-pro-BNP had been used as markers to detect left ventricular dysfunction and to diagnose CHF. The levels of BNP and NT-pro-BNP have strong correlation with the severity of the patient's conditions, which could be used as an indicator to predict possible outcomes of patients with CHF or as a guide to implement appropriate treatment for the patients. Some researchers have mentioned the preference of NT-pro-BNP (half-life 60 to 120 minutes) over BNP (half-life 22 minutes) due to its longer half-life. Also, if BNP is needed for intravenous treatment, usage of NT-pro-BNP minimizes any possible interference of the measurements.

2.2.2 Therapeutic functions and delivery

Natriuretic peptides play concomitant roles in various parts of the body including the cardiovascular, central nervous system and bone. For more details, readers are referred to these excellent reviews [65, 68, 79, 81, 82]. In this section, we focus on discussing the therapeutic functions of natriuretic peptide in cardiovascular regulation and protection.

Endogenous NPs

Apart from playing roles as biomarkers of CHF, circulating natriuretic peptides also play important roles in regulating cardiovascular functions. Endogenous production of ANP is stimulated by increased atrial stretch [83] and overloading conditions [84]. The elevation in endogenous BNP levels is influenced by ventricular wall stress and sodium loadings [85]. Endogenously, pathological conditions such as hypoxia, sepsis and renal failure induce the elevation of CNP [86]. The secretion of CNP is also enhanced by cytokines [87] and growth factors [88]. Moreover, the presence of ANP and BNP in the system also elevates secretion of CNP. In one report, CNP production in aortic endothelial cells was stimulated 400-fold by BNP which was 20 times greater than stimulation by ANP [89].

Studies undertaken to understand the roles of endogenous NPs were largely carried out via genetically knockout mice, where the production of NPs are disrupted or NPRs are deleted [63]. Stevens and co-workers elucidated the role of endogenous ANP as cardiovascular homeostasis regulator via NPR-A antagonist study performed in

canines [90]. In another study performed by the same group of researchers, elevated ANP levels during CHF were found to regulate the cardiorenal functions and cardiovascular hemodynamic [91]. The inhibition of ANP elevation resulted in the reduction in ventricular functions, where mild CHF could accelerate to severe CHF due to impairment of natriuretic and diuretic functions. Researchers also found similar observation in mice with BNP gene knockout, where these mice had fibrotic lesions in their ventricles due to ventricular pressure overload [92]. In other reports, the lack of NPR-A resulted in hypertension, cardiac hypertrophy and sudden death in mice [93-95]. In literature, there were no cardiovascular specific knockout model reports pertaining to the absence of NPR-B and CNP. However, the lack of NPR-B [96] and CNP [97] were reported to result in dwarfism, alter ossification and even caused early death.

Exogenous NPs

The endogenous NP studies give us an understanding on the roles NPs played. In this section, we review the physiological effects exerted by exogenous NPs, with emphasis on CNP.

Studies have shown that exogenous administration of ANP and BNP enhances natriuretic and diuretic responses. In one of the initial studies, infusion of ANP in dogs showed increase in glomerular filtration rate and decrease in re-adsorption [98]. However, the usage of BNP also caused some patients to experience renal resistance [99] and in some cases worsening of renal functions [100]. To circumvent the hypotension, Chen and co-workers have utilized doses which were lower than the conventionally used [101]. They have shown that infusion of low concentration BNP

for 72 hours is well tolerated. However, longer term studies need to be carried out to better understand the efficacy and tolerant levels.

Although CNP does not possess cardiorenal functions, it had been reported to be a potent anti-inflammatory agent, vasodilator and inhibitor [86, 102-104]. Anti-inflammatory and angiogenic properties of CNP were reported in pathological models [105]. In rats induced with myocardial inflammation, continuous infusion of CNP for two weeks preserved cardiac functions and decreased tissue necrosis. As a vasodilator, CNP had shown relaxation up to 46% in canine arteries [106] and 9% in rats cerebral arteries [107]. CNP had also been reported to be a potent inhibitor in both in vitro and in vivo studies. The administration of CNP observed inhibition in serum-induced rat vascular smooth muscle cell [108]. In a study carried out on rabbits [109], infusion of CNP post balloon catheter injury resulted in suppressed neo-intima formation. Moreover, Soeki and co-workers have shown that rats infused with CNP were protected against cardiac remodeling post MI [15] via inhibition of collagen synthesis. However, the infusion of CNP at low doses in healthy subjects were found to have no effect on cardiac outputs and pressure [16], which the authors attributed to low number of NPR-B receptors and guanylyl cyclase activity. Furthermore, continuous supply of CNP had also been reported to be superior over single administration. In CNP gene transfer study, authors observed suppression of neointima formation whilst endothelial healing was not compromised [110, 111].

Routes of administering NPs

In view of the potential advantages of exogenous NPs, the routes of administering NPs have also been extensively researched and over the past decade some researchers had

proposed novel routes. These routes include oral delivery [112, 113], renal delivery [114] and parental delivery [69-71] of NPs.

Oral delivery of biological therapeutics had been challenging due to the difficulties associated with the loss of bioactivity, protein adsorption and penetration barriers. Cataliotti and co-workers came up with the idea of modifying human BNP using a proprietary methodology which bonds BNP covalently to amphiphilic oligomers consisting of alkyl and PEG moieties [112, 113]. The conjugated BNPs were orally administered to normal adult dogs and were found to activate cGMP production and reduce the mean arterial pressure. The performance of the conjugated BNP was also found to demonstrate equal or better response than native BNP, suggesting potential of NP modifications. However, one of the major short-coming mentioned by the authors is the requirement of high dose due to the low bioavailability via the oral route.

The usage of BNP also caused some patients to experience renal resistance [99] and worsening of renal functions [100] due to hypotension associated with systemic administration. Chen et al have proposed the delivery of BNP directly into kidneys to reduce the effects of renal perfusion pressure and eradicate systemic hypotension [114]. They found that local delivery of BNP into the renal system resulted in superior renal responses compared to systemic BNP delivery, which was attributed to the elevated amount of BNP reaching the NPR in the system before clearance and degradation. It appears that the delivery of BNP via the renal route is a promising strategy. However, such organ targeted delivery strategy is limited to renal-enhancing peptides and therapeutics. As such, this strategy may not be useful for CNP which has

no renal enhancing capabilities. Moreover, such applications have low patient compliance and may introduce other complications.

Despite the development of novel routes, the most pursued therapeutic route to delivery NPs is the parental administering of NPs via intravenous or subcutaneous injection of bolus or continuous supply of NPs over time [67, 69, 70, 115]. This route of administration is attractive due to the ease of controlling desired peptide dose and ease of application. In fact, in therapeutic applications, Carperitide (human recombinant ANP) and Nesiritide (human recombinant BNP) had been approved for intravenous administration in acute decompensated Heart Failure (HF) conditions since 1995 and 2001 respectively [69-71]. However, this mode of non-localized delivery is wasteful and non-efficacious [9, 35], especially for pathologies that require localized treatment. Presently, the existing routes for administering NPs appear to be unsatisfactory due to the low efficacy, high costs or low patient compliance. *There is a need for the development of alternative routes to make the administration of NPs more clinically viable.*

2.2.3 Designer peptides

In sections 2.2.1 and 2.2.2, the potential of NPs in treatment of cardiovascular diseases is established. However, the native NPs have their respective inherent undesirable properties. The usage of ANP and BNP induces hypotension and might worsen renal functions; and CNP lacks natriuretic and diuretic properties. These incompatibilities of native peptide had made the use of analogs of native NPs less appealing and have *propelled the development of novel and designer peptides* [13, 74, 116-119].

The birth of novel peptides have stemmed from the motivation to develop ideal pharmacologic profiles. The methods to synthesize novel NPs could be broadly categorized into two— alternative splicing and engineered designer NPs.

Alternative Splicing

The first mode is alternative splicing, where alterations are done at pre-messenger RNA (mRNA) level. Pan et al had synthesized alternative splicing BNP (ASBNP) and a truncated form known as ASBNP.1 [118]. Unlike the natural BNP, both ASBNP and ASBNP.1 failed to stimulate cGMP in human vascular smooth muscle cells. Overall, both ASBNP and ASBNP.1 failed to retain dose-limiting vasoactive properties of BNP. However, in the HF model in canine, the truncated form ASBNP.1 possessed distinct renal effects. The authors concluded that ASBNP.1 may be therapeutically viable in HF patients with cardio-renal syndrome.

Engineered designer NP

The second mode of synthesis is engineering native NPs together to create hybrid peptides. This mode of synthesis was developed before the alternative splicing method. In 1993, the first hybrid known as vasonatrin (VNP) was created by Wei et al, where the entire 22-AA CNP was fused with 5-AA carboxyl terminus of ANP [120]. VNP was found to possess natriuretic and diuretic properties greater than CNP. More importantly, VNP retained the beneficial renal effects of CNP while exhibiting unique vasodilation properties of ANP. The other peptide synthesized by this method is CD-NP, which will be reviewed in the next section.

2.2.4 CD-NP (Cenderitide)

C-type natriuretic peptide (CNP)

Amongst the NPs, CNP is the most highly conserved [102]. From CNP's 126-amino acid preprohormone, two mature forms of CNP are derived—CNP-22 and CNP-55 [121]. As CNP-55 is predominantly stored in tissue whilst CNP-22 circulates in plasma, hence most of the reports on biological effects is related to CNP-22 [102]. Despite the huge potential CNP possessed, it is very much limited by its short half-life of approximately 2.6 minutes [122]. Also, the lack of natriuretic and diuretic properties has been a major limitation to its usage.

CD-NP

In view of the mentioned problems, CD-NP was synthesized by the Mayo Clinic [13]. There are two forms of CNP and CD-NP originates from CNP-22. It is a hybrid of native CNP from human and a C-terminus isolated from the *Dendroaspis* natriuretic peptide found in the venom of the green mamba [13, 74, 116]. Figure 2.4 shows an illustration of the CD-NP structure. By combining the ideal pharmacological profiles of native peptides, CD-NP possesses complementary diuretic and natriuretic properties of DNP and reduced hypotensive property of CNP [13].

Furthermore, the addition of DNP converted non-agonist of GC-A characteristic of CNP to partial-agonist of GC-A. Its ability to activate GC-B is derived from its CNP origin; such activation reduces cardiac load through venodilation and reduces cardiac hypertrophy and fibrosis. The addition of DNP did not result in loss of ability to activate GC-B, which allows the regulation of natriuresis and diuresis [68, 74, 75].

Hence, CD-NP is the first NP that possesses dual activation of GC-A and GC-B [75, 123, 124]. Apart from additional functionality, the addition of the C-terminus tail had resulted in higher resistance to proteolysis, which increased the half-life of CD-NP and prolonged its biological actions [74].

The parenteral administration of CD-NP had been reported to be effective against cardiac remodelling [14]. Burnett and co-workers have shown that intravenous and subcutaneous infusion of CD-NP reduced the LV mass in myocardial infarction (MI)-model rodents, exhibited cardiac unloading in dogs [13] and induced natriuretic and blood pressure responses in humans [14]. CD-NP also possessed anti-proliferation properties, where it was reported to be effective in preventing restenosis via the inhibition of human aortic smooth muscle cells (HAoSMCs) proliferation [125].

The promising pre-clinical findings also prompted clinical trials study. The first-in-human clinical trials were carried out by Lee and co-workers [126]. The trial was carried out in two stages. In stage 1, a dose-escalation study was carried out. Patients were intravenously infused with CD-NP at dose of 10, 17.5 and 25ng/kg/min. Hypotension was observed in 2 out of 4 subjects at the highest dose and the maximum tolerated dose was set at 17.5ng/kg/min.

In stage 2, the double-blind study was carried out. Subjects infused with CD-NP at 17.5ng/kg/min for 4 hours were compared to subjects that were given placebo treatment. In both groups, the glomerular filtration rate (GFR) and mean arterial pressure (MAP) were found to be similar. However, in the CD-NP treatment group, plasma and urinary cGMP levels were found to be significant compared to placebo

group. Moreover, urinary sodium excretion and urine flow were found to be significant. The authors concluded that CD-NP clinical trials achieved overall favorable cGMP-stimulation, natriuretic responses and absence of excessive hypotension in healthy adults.

Figure 2-4 Structure of CD-NP [13]

2.2.4 Challenges of delivering NP

Despite considerable research in this area, two major challenges remain as stumbling blocks. They are the *inherent nature of the NPs* and *difficulty in routing NPs to the site of need*.

Short circulation half-live and intrinsic hydrophilicity

Exogenously NPs have exhibited beneficial responses at appropriate doses; however their its short half-live greatly limits its therapeutic use. NPs have very short in vivo half-live, with BNP having the longest half-live of 20.3 minutes. The short biological half-live makes it essential yet difficult to maintain within the therapeutic window

levels, particularly for chronic treatment. In this context, multiple dosing or continuous supplies are essential in order to achieve sustained therapeutic effect. Furthermore, the intrinsic hydrophilicity of the NP makes its encapsulation in hydrophobic matrix difficult.

Route of administering

As mentioned earlier, the only approved means of delivery of peptide is via intravenous injections and subcutaneous infusion [67, 69, 70, 101, 115, 127, 128]. However, these modes of systemic delivery involve elevated dosages and frequent injections, resulting in therapeutic wastage and low compliance. It seems that a localized delivery system would attenuate all the problems associated with systemic delivery. *It is apparent that the delivery of CD-NP from devices and platforms would enable the peptide to be delivered locally and continuously.*

2.3 Biodegradable polymers

Section 2.3 is sub-sectioned into five sections. In section 2.3.1, the relevance of biodegradable polymers in biomedical device is reviewed. Section 2.3.2 and 2.3.3 reviews the mechanism of polymer degradation and the factors that affect polymer degradation respectively. Section 2.3.4 and 2.3.5 reviews the polymers used in this work which are PCL and PEG.

2.3.1 Biodegradable polymers and biomedical devices

Biodegradable polymers have been widely used in many applications ranging from cornstarch based 'green' utensils, waste bags to soy protein based automobiles parts produced by Ford. By and large, the usefulness of biodegradable polymers is no doubt vast and extensive. Particularly in the past decades, biodegradable polymers had received a lot of focus and attention in the field of biomedical applications. In fact, the use of biodegradable polymer for medical application could be traced back to the Roman's time where catgut was used in surgical sutures.

When we consider the use of biodegradable polymers as material candidate for biomedical devices, they need to be compatible and reliable for use in human body. The following are some of the desirable characteristic:

1. Biodegradable

The polymer needs to be biodegradable if it is intended for temporary intervention procedures and usage. As these biodegradable polymers will eventually metabolize in the body, the hassle of a second surgical intervention could be eliminated.

2. Biocompatible

Since it would be implanted in vivo, the polymer should be well tolerated in the body and not evoke inflammatory responses. Hence, the choice of non-toxic and biocompatible polymers is essential.

3. Well-defined

For biomedical devices, the reproducibility in terms of their degradation and performance is crucial. In this case, the use of synthetic polymers would be superior over the use of natural polymers due to greater control of polymer quality and properties. For instance, the rate of biodegradation of synthetic polymer could be adjusted by modulating its molar mass, composition and crystallinity. The classic example of such a synthetic polymer is poly (lactide-co-glycolide) (PLGA). PLGA had been widely studied and a number of biomedical devices made of PLGA had been approved by the Food and Drug Administration (FDA). By modifying the percentage of lactide and glycolide composition, it is possible to achieve the desired degradation profiles and even control the therapeutic release profile based on their known degradation behavior [129-133].

4. Sterilization

Most of the medical devices are required to be sterile before implantation. In the past, high temperature sterilization processes restricts the use of low melting point polymers as material. However, with the emergence of room temperature ethylene oxide (ETO) sterilization, the choice of polymer is no longer restricted by their thermal properties.

5. Physical attributes

Depending on the application, the physical attributes of the polymers should be chosen accordingly. If the polymer is needed as physical support, the mechanical properties of the polymer need to be considered. In ocular devices,

the translucency and flexibility of the polymer would be relevant factors to consider.

Apart from the attributes listed above, the feasibility of molding or shaping the polymer into the desired device should also be considered. In the next few sections, the degradation mechanism of biodegradable polymers and the polymers used in this study would be reviewed.

2.3.2 Polymer degradation mechanism

In the macro-level, polymer degradation refers to the “break down” or the reduction in size of a polymeric bulk. In the molecular level, polymeric degradation refers to the cleavage of monomers from the polymeric chains. Different biodegradable polymers can undergo a myriad of degradation such as enzymatic, thermal and mechanical degradation.

In this section, we focus on polymers that undergo hydrolytic degradation. Hydrolytic degradation is associated with the breakdown of polymer in the presence of water. And hydrolytic degradation could be broadly categorized into “surface erosion” and “bulk erosion” [134, 135]. “Surface erosion” is a heterogeneous process where the rate of polymer hydrolysis is faster than the rate of water penetration into the matrix. On the other hand, “bulk degradation” is a homogenous process where the rate of water penetration into the matrix occurs more rapidly than the polymer hydrolysis. Figure 2.5 describes these two processes in a pictorial manner. Both types of erosion have

their respective advantageous and disadvantageous, hence the choice of polymer should be made based on the application needs.

Figure 2-5 Schematic describing surface and bulk erosion in biodegradable polymers

2.3.3 Factors that affect polymer degradation

There are numerous parameters that affect the erosion of biodegradable polymer and the following are some of the factors [136] (the list is non-exhaustive):

1. Chemical composition

Depending on the polymer type, intrinsic viscosity and composition, the degradation could vary from days to years [137].

2. Molecular mass

The biodegradability of polymers is inversely related to its molecular mass. In low molecular mass polymers, the carboxylic and hydroxyl end group content is relatively high, and the presence of carboxylic groups induces autocatalysis.

3. Presence of plasticizers

The presence of residual solvents and drugs can act as plasticizers and dramatically affect the degradation behavior of polymers. Also, the presence of acidic and basic drugs could enhance or decline the degradation rates of polymers.

4. Crystallinity

In semi-crystalline polymer, the degradation commence from the amorphous region before proceeding to the crystalline regions. This implies that polymer with higher crystalline observed a lower polymer degradation rate [137, 138].

5. Storage conditions

Storage condition such as temperature, humidity and pH affects the degradation of polymers. And the presences of heat, moist and acidic environment would accelerate the degradation of polymers.

2.3.4 Poly (ϵ -caprolactone) (PCL)

Figure 2-6 Chemical structure of PCL[145]

Synthesis and properties

Poly (epsilon-caprolactone) (PCL) is a semi-crystalline polyester which is synthesized by open-ring polymerization. Its melting point falls in the range of 59 °C to 64 °C and it has a low glass transition temperature of -60 °C [139], which gives it a rubbery property at room temperature. PCL and its copolymers degrade by bulk hydrolysis in two phases. In the first phase, random hydrolytic chain scission occurs resulting in the reduction of its molecular mass; and in the second phase, smaller fragments are broken down resulting in the decrease of molecular mass [140]. Complete degradation of PCL is recorded to last 2 to 4 years [141, 142]. The presence of hydroxyl radicals is likely to accelerate the rate of degradation.

Extensive toxicology of PCL had been studied and its degraded monomeric hydroxyacid was found to be biocompatible with tissue and rendered non-toxic. PCL exhibits good biocompatibility, biodegradability and is commercially available. These desirable characteristics make it an ideal candidate for diffusion controlled drug delivery [143, 144]. In fact, a handful of PCL fabricated drug delivery devices have already received FDA approval and CE Mark registration [145]. However, in the literature, controlled release of protein and peptide from poly (lactide-co-glycolide) were more extensively reported [129-133] compared to PCL. This is probably due to slow release of protein from PCL compared to the PLGA polymers. PCL displayed slower release kinetics due to the presence of high crystalline domains that inhibit the water penetration into the segment [137]. Pitt and co-workers reported that PCL with molar mass of 5000 Da has approximately 80% crystalline, while PCL of 60,000 Da has approximately 45% crystalline [138].

In view of the slow release, PCL had been modified via chemical and physical means to boost the release rates or obtain the desired release profiles. The chemical means involves the usage of PCL-type copolymers whilst physical means involves the usage of less crystalline polymers as blends.

Copolymers

Kim and co-workers modified the PCL segment, synthesizing mPEG-PCL diblock and explored the release from the matrices. They tested release of bovine serum albumin (BSA) from diblocks of different composition. The diblock formulations retained a similar profile as PCL showing an initial burst and the amount of BSA release increase with the decrease in PCL segment. The authors attributed this to the relative increase in mPEG segment which resulted in improved water-penetration into the system [146]. Similarly, other groups also showed that PCL copolymer degrades more rapidly compared to homo-polymer PCL due to decrease in crystallinity [137].

Blends

The rational for polymer blending is to modify the matrices properties, which in turn alters the drug release. The addition of blends is a simple way to alter the degradation and release rates from PCL. Several authors have worked on blending PCL with new polymers [147], polycarbonates [148] and other polyesters[149-151]. In one study, McDonald et al studied the degradation and drug release of thin PCL films blended with poly(DL-lactide) (PDLLA) and PLGA83/17 [150]. The presence of PDLLA and PLGA accelerated the degradation of amorphous regions in PCL and the authors managed to attain varied release profiles by changing the ratio of the blends. In a separate report by Shen et al, they reported that apart from composition, the

compatibility of blends is an important factor that affects degradation and release [151]. Compatibility of blends was investigated using the differential scanning calorimetry (DSC). Incompatible blends observed faster release compared to compatible blends, which was attributed to the release of drugs from micro channel formed due to phase-separation.

Blending with ethylene-based moieties has also been reported. Huatan and co-workers studied the effects of PCL blended with poly(ethylene oxide) based-copolymer [144] on the release of BSA. They found that the addition of the copolymer increased the release of BSA due to the increased in water content and permeability. Jones and co-workers also reported enhanced release upon addition of PEG, which they attributed to enhanced porosity due to the addition of PEG [152].

2.3.5 Poly (ethylene glycol) (PEG)

Figure 2-7 Chemical structure of PEG

Synthesis and properties

PEG is a polyether terminated with a hydroxyl end group. It is synthesized via ring opening of ethylene oxide and has a general formula as shown in figure 2.7. In comparison to other polymers, PEG possess a relatively narrow polydispersity range of 1.01 for low molecular weight (for $M_w < 5$ kDa) to 1.1 for high molecular weight

(for $M_w > 50\text{kDa}$) [153]. PEG is a unique molecule that is able to solubilise in both aqueous solution and organic solvent [153]. Due to PEG's highly flexible backbone chain, it is able to bind with water molecules as if it were five to ten times the size of a soluble protein of equal molecular weight [153].

Usage

PEG dissolute rapidly in vitro and in vivo, hence it cannot be directly used to form matrices for controlled release. Unlike typical biodegradable polymers used, PEG has been extensively used both to enhance the polymer system and the therapeutic agents itself. The usage of PEG could be broadly differentiated into physical [154, 155] and chemical [133, 153].

Chemical perspective: PEGylation

Since the 1950s, PEGylation has been known and practiced [156]. PEGylation is a process where PEG conjugates to proteins and peptides; it involves the covalent bonding of a functionalized PEG polymer to the N-terminal amino group or C-terminal carboxylic acid group on the polypeptide of interest. Since the work on PEGylation's enhancing effects on the circulating life and pharmaceutical properties [157] have been published, there have been a lot of research on elucidating PEGylation's ability to prolong the circulation times of conjugated therapeutic. Today, a handful of PEGylated therapeutics had been approved by the FDA. PEGylated therapeutics is not cleared by the kidney due to their increase in hydrodynamic radius and the formation of protective shell, which prevents uptake by the reticulo-endothelial cells. For instance, upon the PEGylation of 5 kDa PEG to the G120K-Growth Hormone (GH) antagonist, the half-life increased from 30 minutes to more than 100

hours [158]. However, readers should be brought to attention that steric interference as a resultant of excessive PEGylation may result in loss of therapeutic activity. And for the case of the G120K-GH antagonist, the extension in half-life resulted in reduction in potency.

Chemical perspective: Copolymer

Bezemer and co-workers developed multi-block copolymer based on PEG and poly (butylene terephthalate) (PBT) [155] for the release of model protein. This copolymer had unique property consisting of ‘hard’ domains due to the PBT and dispersed ‘soft’ domain due to the PEG segments. They found that the degree of polymer swelling was dependent on the PEG length. Moreover, the rate of lysozyme release was dependent on the PEG content, which meant that increase in molecular weight of the PEG segment resulted in faster release rate [155].

Physical perspective: As excipient

In highly crystalline polymers, the diffusion of therapeutic through these polymers had been too slow for practical usage. The usage of excipients in protein and peptide release had been incorporated for various reasons, which includes improving the peptide bioactivity [129, 159-161], enhancing matrix properties [162, 163] and assisting complete release [164-168]. Due to the flexibility and hydrophilicity, the usage of PEG and PEO as excipients have been extensive.

PEG is widely used as excipient to regulate drug release in polymeric matrices [13, 76, 116, 123, 129, 160, 162-171]. From literature, PEG has been widely reported to accelerate or amplify the release of proteins and drugs [13, 76, 131, 160, 164, 167-169,

172]. The increase observed had been largely attributed to the increase in porosity or loosely bound peptide at the surface [143, 165, 166, 173]. There had been few reports on PEG decreasing initial release [165, 166] and the authors attributed the observation to altered solubility or reversible precipitation of drugs in the presences of excipients. However, in these studies, the addition of excipients eventually led to higher release compared to formulations without excipient addition.

PEG and PEO had been reported to play roles of as emulsifier and surfactant in polymer systems. A study carried out by Park et al, the group showed that addition of poly (ethylene oxide) (PEO) improved the system stability and they concluded in their study that higher concentration of PEO was able to produce more stable-form of microcapsules [168]. Apart from its ability to stabilize the system, PEG also provided 'protective effects' to the protein during delivery. Pean et al and Bezemer et al found that the addition of PEG had stabilizing effect on encapsulated protein and PEG also has the ability to de-anchorage protein from polymer matrix [154, 155]. Pean et al used PEG 400 as an excipient to improve the stability and release of NGF from biodegradable microspheres. The addition of PEG induced protein de-solvation, minimized protein denaturation and reduced the protein anchorage in the polymer layer, hence preventing "level off" in release profile [154].

2.4 Peptide and protein delivery

Proteins and peptides essentially participate in all biological processes that occur in our bodies. In the field of drug delivery, they are in vogue due to their highly specific

structures, compatibility with the human body and complex functions that synthetic drugs had been incapable to mimic. Furthermore, the likelihood of proteins and peptide interfering with the body's biological processes and giving rise to toxicity issues are sparse.

Since the discovery of insulin in 1922 [174], the scientific and medical communities have been searching for means to delivery exogenous proteins and peptides in a satisfactory manner. However, it was the development of recombinant DNA technique to produce human recombinant protein that changed the entire trajectory of the field. In 1977, Boyer and co-workers produced the first human recombinant peptide, somatostatin, through the insertion of recombinant DNA into an *Escherichia coli* (*E. Coli.*) [175]. By 1982, the first human recombinant therapeutic, insulin, was approved by the FDA[176]. The other method that is extensively used today is the solid phase protein synthesis. This method involves the usage of a functionalized resin bead for a growing peptide chain to grow via cycles of “activation” and “deprotection” depending on the ultimate length of the chain. The human recombinant NPs described in section 2.2 were produced by this method. These two methods allowed the up scaling on production of peptides and in 2008, one out of five blockbuster drugs is a protein or peptide based therapeutic.

Despite the advancement in the purification and production of peptide, challenges in formulating, storing and delivering them effectively remains. In sections 2.4.1 and 2.4.2, we review the challenges faced when delivering proteins and the advances in the protein and peptide delivery systems. Finally, in section 2.4.3, we explore the use of polymer deport systems to delivery peptides.

2.4.1 Prospects and challenges of protein delivery

Proteins and peptides are very sensitive to the environmental changes and factors. Due to the delicate nature of proteins and peptides, a lot of difficulties were faced during the formulation and storage processes as they are high susceptible to **physical and chemical instability**. During formulation, protein or peptide exposure to shearing, elevated temperature or change in pH could result in aggregation and precipitation. The aggregation of the proteins may have a direct impact on the potency or immunogenicity. Both proteins and peptides are susceptible to the effects of chemical degradation from hydrolysis, deamination and oxidation. The effects of physical instability are more prominent in proteins compared to peptide due to their higher tertiary structures. This is because any conformational rearrangement of the 3 dimensional structures could result in the loss of biological activity. Moreover, they have **short plasma half-lives**. This could be attributed to their cellular involvement, including receptor mediated endocytosis. As mentioned in the earlier section, NPs are removed from system via clearance receptor, NPR-C. Also, circulating NPs are susceptible to neutral endopeptidases (NEP) degradation, where they are cleaved and inactivated [177].

The challenges faced spurred a lot of research ideas and efforts, in attempt to sought solutions to overcome them. And they would be reviewed next in section 2.4.2.

2.4.2 History and developments of protein and peptide delivery

To counter the challenges mentioned in section 2.4.2, much effort had been invested in two prongs—(1) direct modifications to the proteins or peptide and (2) innovation on delivery of these therapeutics.

Modifications to the proteins and peptides

In the first prong, groups attempted to prolong the circulation time of therapeutics by decreasing their susceptibility from being cleared by the kidney or undergo proteolysis. This largely involves conjugating proteins or peptides via PEGylation or hyperglycosilation, which changes their confirmations and binding properties. PEGylation involves the conjugation of PEG to proteins. (Kindly refer to section 2.3.5 for details) On the other hand, glycosilation involves the attachment of oligoscaharides to proteins. Despite the difference in the two processes, their rationales and goals are similar, that is to achieve extended biological half-life and reduce immunogenicity. Unfortunately, the pitfall for these modifications lies in the unpredictable conformational changes, which may lead to loss of potency.

Innovating the means of delivery

In the second prong, work has been focus on diversifying the delivery routes and fine tuning current processes in attempts to increase bioavailability and retain the bioactivities. A review of the various strategies categorized by the route of delivery would be reviewed.

(1) Oral

From the patient's point of view, oral administration of medication is perhaps one of the simplest and least intimidating medical options. In fact, 80% of small molecule drugs are delivered via tablets and capsules [178]. Yet from a pharmacologist point of view, formulating oral therapeutics is challenging, especially protein and peptide therapeutics. This is because the gastro-intestinal (GI) tract consists of treacherous barriers, including acidic environment of the stomach, enzymatic degradation and low permeability of drug through GI membrane.

The use of enteric coatings and enhancement of the therapeutics itself had received considerable attention in view of the problems. The rationale of enteric coating is to protect encapsulated therapeutic agents from the acidic environment in the stomach and subsequently releasing them in the upper intestines [179]. Cellulose based polymers and polyvinyl derivatives are commonly used for enteric coatings because their thickness and threshold dissolution pH could be adjusted and thus the location of release within the GI tract. In section 2.2, we mentioned the use of proprietary technique to bond BNP covalently to amphiphilic oligomers consisting of alkyl and PEG moieties [112, 113]. The use of proprietary enhancements had also been reported in the delivery of insulin. In one study, the authors reported that the potency of 980 units of insulin delivered orally was equivalent to 8 units of insulin delivered subcutaneously, suggesting that the bioavailability of oral delivery was merely 0.8% compared to subcutaneous injections [180]. Although the non-invasive nature of oral delivery is very attractive; however the low bioavailability continues to hamper its usage.

(2) Parenteral

Parenteral routes are advantageous over oral routes due to the high bioavailability and prompt commence of action after administrating. Broadly, parenteral administration could be categorized into three types, depending on their onset time [181]. For immediate results, the *intravenous administration* is used, while *subcutaneous or intramuscular injections* are used for sustained delivery. For peptide delivery, intravenous and subcutaneous injections are more commonly used.

The intravenous route of administration is attractive because desired dose could be controlled and administration could be halted immediately if adverse effects were observed. The intravenous administration of human recombinant ANP and BNP has been used for the treatment of acute decompensated HF [69-71]. However, despite the prevalent usage of parenteral administration, there are many shortcomings associated with it. Adverse reactions at injection sites including nodules and erythema accounts for 53% and 61% of occurrence in injected patients respectively [176]. Moreover, this mode of delivery is wasteful, non-efficacious and low in patient compliance. Since it has to be done in a hospital setting, it is expensive and the patient is also immobilized during the period of administration.

In view of the limitations, implantable pumps with drug reservoirs were introduced for long-term usage [182]. The usage of these pumps eliminates the problems associated with needle usage complications and increases patient's mobility. However, safety issues including system instability and the lack of control after implantation have been the major limitation. Moreover, the need of surgical intervention for the insertion and removal of the device makes this method less desirable.

(3) Pulmonary

Pulmonary delivery of proteins and peptide presents an alternative route for powdered formulation delivery. For pulmonary delivery to be effective, the essential criterion is for the drugs to reach deep lungs. Hence, the therapeutic agents size needs to fall within the range of 1-3 μ m and the range might be more stringent for certain therapeutic agents [176]. The use of pulmonary administration to deliver insulin had been reported to possess approximately 10 % bioavailability compared to subcutaneous administration [183]. However, there are safety concerns associated with damage of lung tissues due to long term usage, which needs to be verified.

(4) Others

Other delivery routes via rectal, vaginal and ocular systems have also been investigated. The main advantage of these routes is that they bypass the hepatic metabolism. Moreover, these delivery routes are particularly advantageous for specific pathology that requires localized delivery. For instant, liposome [184] and micro-films [185] encapsulating therapeutics had been investigated to reduce intraocular pressure and reduced inflammation in glaucoma-model rabbits respectively. Both studies have shown that sustain-release technologies via alternative routes are effective. In the next section, we review the use of sustained-release systems for protein and peptide delivery.

2.4.3 Polymeric sustained-delivery systems

Polymer-based protein delivery systems were initially pioneered by Professor Robert Langer and co-workers to address the issue of short half-lives of proteins. The incorporation of proteins and peptides directly into the polymer matrices during the manufacturing process is a direct approach for preparation of delivery devices. Depending on the desired porosity and thickness of polymeric matrices, they could be manufactured by solvent casting, spin-coating and injection molding. Polymeric delivery systems to control release of peptides could be categorized as diffusion-controlled, chemically-controlled, solvent activated and stimulus triggered release [186]. Solvent-activated and stimulus triggered systems are out of the scope of this work. In the following we discuss how variation of release could be obtained from diffusion-controlled and chemically controlled systems.

A diffusion-controlled system refers to a non-swelling polymer system (non-biodegradable or bio-stable) where release is dependent on the diffusivity of peptide out of the system. A chemically-controlled system refers to systems where peptide release is dependent on the mechanistic of polymer degradation. The rate and mode of polymeric degradation (bulk and surface erosion) are means to control the release rates and kinetics.

To achieve release profile variations in these systems, blending with polymers [143, 144, 187] and the addition of excipient [131, 188-190] are frequently employed. Readers are referred to sections 2.3.4 and 2.3.5 for more details on these methods. In the recent years, there have been new interest developed in the field of phase inverted membranes as a means to control therapeutic agent release [191, 192]. Phase inversion occurs in immiscible liquid-liquid dispersion where initially a homogenous single-

phase is formed when polymer is dissolved in solvent and non-solvent (most likely containing drugs compounds) or additives are added to induced phase separate via solvent-non-solvent exchange [193]. The membrane formation effects of phase inversion had been studied [193, 194]. In two studies carried out by McHugh et al, they gave insights on how the process, solvent composition could immensely affect the membrane outcome and drug delivery, which enable some degrees of success in design of membrane-based drug delivery systems [191, 192].

Chapter 3 Experimental Materials and Method

3.1 Materials

The materials used in this study are listed in the following, Table 3.1. All organic solvents used were of HPLC grade and were used as received.

Table 3-1 Table of materials and reagent

Materials	Abbreviation	Company
<i>Polymer</i>		
Poly (ϵ -caprolactone), Mn 80,000 g/mol	PCL	Sigma Aldrich
Polyethylene glycol, Mw 2,000 g/mol	PEG	Merck-Schuchardt
Poly (ϵ -caprolactone) (1.5K)-Polyethylene glycol diblock copolymer	PCL-PEG	Avanced Polymer Materials Inc.
<i>Peptide/ Cytokine</i>		
Chimeric fusion of C-type Natriuretic peptide and C-terminus of Dendroaspis Natriuretic peptide	CD-NP	American Peptide Company
Human Cardiotrophin-1	CT-1	GenWay (USA)
<i>Organic Solvent and Acid</i>		
Dichloromethane (HPLC grade)	DCM	Tedia Chemical Company
Acetonitrile (HPLC grade)	ACN	Tedia Chemical Company
Trifluoroacetic acid (HPLC grade)	TFA	Tedia Chemical Company

Detection Assay

micro-Bicinchoninic Acid protein Assay Kit	BCA	Thermo Scientific
cGMP Assay	-	GE Health Company
Cell Proliferation ELISA, BrdU	BrdU	Roche Applied Science
Buffer solution, pH 7.4		OHME Scientific

3.2 Methods

3.2.1 Film preparation

The polymer solution was prepared by dissolving 0.9 g PCL pellets in 5 mL of DCM and stirred continuously to obtain homogenous solution. The peptide solution was prepared separately, by dissolving 1 wt% of CD-NP in water or ethanol according to their co-solvent system formulation. Subsequently, the polymer solution and peptide solution were added together and emulsified, where the emulsification duration was monitored. The following table 3.2 shows the respective peptide solution and polymer solution composition for each formulation.

Table 3-2 Film formulation and peptide loading

Co-solvent System	Formulation	Polymer solution	Peptide solution
DCM/Water		PCL + DCM + ..	CD-NP + Water + ..
	PCL	Nil	Nil
	5% PEG	Nil	5% PEG

Chapter 3 Experimental Materials and Method

10%PEG	Nil	10% PEG
5% PEG-PCL	5% PEG-PCL	Nil
10% PEG-PCL	5% PEG-PCL	Nil
DCM/Ethanol	PCL + DCM + ..	CD-NP + Ethanol + ..
PCL	Nil	Nil
5% PEG	Nil	5% PEG
10%PEG	Nil	10% PEG
5% PEG-PCL	5% PEG-PCL	Nil
10% PEG-PCL	5% PEG-PCL	Nil

Protein loaded polymer solution was casted on glass plate using an automatic film applicator (Sheen 1137). To obtain a final thickness of 40 μm , the adjusted height of casting knife was set at 0.7 mm (for the DCM/Water co-solvent system) and 0.3 mm (for the DCM/Ethanol co-solvent system). The films were then dried under ambient conditions overnight before finally drying in the 37 $^{\circ}\text{C}$ vacuum oven for 1 week. Solvent residue analysis was conducted with Thermogravimetric Analyzer, TGA (TA Instruments Q500). The following steps were taken during the process: samples (~10 mg) were heated from room temperature to 135 $^{\circ}\text{C}$ at 10 $^{\circ}\text{C}/\text{min}$ in nitrogen environment, after which it was held isothermally for 30 minutes. The resulting weight loss revealed < 0.5 wt% residual solvent in all samples. The thickness of the dried films was measured using a micrometer. Fabricated films show a mean thickness of 0.045 ± 0.005 mm (average of at least 5 measurements).

3.2.2 Surface morphology

Samples were dried in 37 °C vacuum oven prior surface morphology study. Samples were gold coated using the sputter for 20 s under argon atmosphere. Samples underwent analysis using the Scanning Electron Microscopy (SEM) (AS JEOL, 6360) operated at 3kV.

3.2.3 Confocal laser scanning microscopy (CLSM)

CD-NP loaded films were analyzed with the CLSM (Leica Lasertechnik GmH, Heidelberg, Germany) equipped with Argon (PMT approx. 600), Krypton (PMT approx. 800) laser. DD 488,514 filters were used. The distribution of CD-NP, PEG and copolymer (PCL (1.5K)-co-PEG (5K)) were observed. Not all molecules are susceptible to excitation by laser but fortunately the component of interest possessed this property and component of interest did not overlap [132]. The auto-fluorescence of CD-NP was excited at 514 nm (gain 750 V); whilst the auto-fluorescence of PEG and the copolymer were excited at 405 nm (gain 650 V). And the resulting emission was 524 to 604 nm for CD-NP and 425 to 485 nm for PEG and PEG-PCL respectively. Table 3.3 is a summary of their respective excitation and emission wavelength. To eliminate emission cross talk, sequential mode was used. No detectable cross talk observed for these experiments.

A depth (z-axis) scan was carried out with the step-size of 0.13 μm and the scanned area of 246 μm by 246 μm . Each image is an optical section. And the summation of all sections were saved as a stack. A total of 300-350 images were collected for each scan depending on the thickness of each specific position.

Table 3-3 Excitation and emission wavelengths of molecules

Excitation Wavelength	Emission	Detection
405 nm (Gain: 650V)	425-486 nm	PEG [blue], Copolymer PCL-PEG [blue]
514 nm (Gain: 750V)	524-604 nm	CD-NP [green]

3.2.4 In vitro peptide release

1 cm × 1 cm films in sets of triplicate was placed in 10ml phosphate-buffer saline (PBS pH 7.4, 37 °C) for the first time point (6 hours) and 3ml PBS was refilled for the subsequent time points (daily for the first 3 days and every 3 days for all subsequent time points till the end of the release study) to maintain sink condition. The protein concentration of the release medium was tested using the micro-Bicinchoninic acid (BCA) protein assay.

3.2.4.1 micro-Bicinchoninic acid (BCA) protein assay

The micro-Bicinchoninic Acid (BCA) protein assay from Pierce was used to quantify the total amount of protein present. BCA utilized colorimetric changes for detection. The detection involves 2 main chemical steps. Step 1 involves the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (biuret reaction). In step 2, it involves the chelation of Cu^{1+} and two molecules of BCA, which results in the formation of a purple-coloured complex. The complex exhibits strong absorbance at 562 nm and the protein concentration detected is proportional to the amount of reduction that occurred

(in step 1). It has been reported that macromolecular structure of protein, several peptide bonds and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) is responsible for the colour formation when protein is assayed with BCA.

The BCA method was chosen due to its compatibility with ionic and non-ionic solvent, stability and broad linear range [195, 196]. Following the PIERCE micro-BCA protein assay protocol, working reagent of MA, MB and MC were mixed in the ratio of 50:48:2. The working reagent was subsequently added to protein samples in a 1:1 volume ratio and incubated at 60 °C water bath for an hour. After incubation, the absorbance of the protein concentration was detected using a UV-Vis spectrophotometer (UV-2501, Shimadzu) and the concentration is calculated based on a calibration curve (figure 3.1). The calibration curve shows the relationship between the known peptide concentration and absorbance value detected between 0-25 µg/ml of peptide concentration.

Figure 3-1 Calibration curve of CD-NP in buffer solution (pH 7.4) using BCA

3.2.4.2 Peptide residual test

A peptide residual test was conducted at the end of the release study. Films were taken out at the last time point, rinsed thoroughly with distilled water and dried in the 37 °C vacuum oven for 7days. The resulting dry weight was taken note and the film was dissolved in 2ml DCM. After 1hr, subsequently 2ml PBS was added and vortex for 10min. The emulsion (DCM/Buffer) was poured into a separating funnel and left to stand still for 1 hr before filtering out the DCM phase. The method was based on the knowledge that almost 100 % of the peptide partition towards the buffer phase in presence of the both DCM and buffer. The buffer solution is then collected and analyzed with the BCA kit. Finally, the residual protein was taken into account along with the total protein released and used to normalize the release profiles.

Calibrations comparisons had been carried out and it had been proven that almost 100% of the CD-NP would partition into the buffer phase. In the following figure 3.2, the graph shows the calibrations carried out.

Figure 3-2 Calibration curve of CD-NP in buffer solution (pH 7.4) after partition in DCM:Buffer (50:50) using BCA.

3.2.5 Polymer degradation

The degradation study was carried out in similar fashion to the release study, 1 cm × 1 cm films in sets of 3 were prepared and placed in 10ml PBS for the first time point (6 hours) and 3ml PBS was refilled for the subsequent time points. Films were taken out at predetermined time points, washed with distilled water and dried in the 37 °C vacuum oven for 7 days. The mass loss and molecular mass changes were used to characterize the degradation of the polymer.

3.2.5.1 Mass loss

The degradation study was carried out in a manner comparable to the release study. At designate time-points, samples were collected instead of the peptide solution. Samples were rinsed with DI water and dried in 37 °C vacuum oven before characterization.

The mass change of the film during degradation was determined by taking the difference of the initial mass of film prior to degradation (M_i) and the mass of film at the designated time points (M_f). The percentage mass loss of the polymeric films was calculated using the following formula:

$$\% \text{ Mass Loss} = \frac{M_i - M_f}{M_i} \times 100\%$$

Where,

M_i is the initial mass of the film before degradation

M_f is the final mass of the film after it underwent degradation

3.2.5.2 Molecular mass analysis

The degree of degradation was monitored via the changes in molecular mass which was measured using the gel permeability chromatography (GPC), Agilent Series 1100 GPC. All samples were dissolved in chloroform at the concentration of 1 mg/ml, filtered through 0.22 μm disposable syringe filters and eluted through a PLGel MIXED-C Column (300×7.5 mm, Agilent) at a flow rate of 1 ml/min. The primary calibration curve was obtained using the polystyrene standards ranging between M_w of 165 to 5000 g/mol

3.2.6 Cell culture

Human cardiac fibroblast (HCF) culture

Human cardiac fibroblast cells were purchased from lonza. Cells were grown and maintained using FGM® medium consisting of recombinant human insulin, r-Human fibroblast growth factor-B (rhFGF-B), gentamicin sulfate amphotericin-B (GA-100) and 10% fetal bovine serum (FBS). Cells were cultured in atmosphere 5% CO₂ in a 37°C incubator. Only cells from passage 3 to 5 were used for this experiment.

Human Coronary Smooth Muscle Cells (HCaSMCs) culture

Human coronary smooth muscle cells were purchased from Lonza. Cells were grown and maintained using the SmGM-2® medium consisting of insulin, human recombinant epidermal growth factor (rhEGF), r-human fibroblast growth factor-B (rhFGF-B), gentamicin sulphate amphotericin B (GA-100) and 5% of fetal bovine serum (FBS) in atmosphere 5% CO₂ at 37°C incubator. Only cells from passage 3 to 6 were used for this study.

3.2.8.1 cGMP production

To investigate the cGMP levels in control and treatment groups, the Amersham cGMP enzymeimmunoassay biotrak (EIA) system was used. The principle for detection is competitive binding, where unlabelled cGMP (produced when NP binds to NPR) competes with a fixed quantity of peroxidase-labeled cGMP (provided by the assay) for binding sites that had been pre-coated on the microplates.

For the detection of cGMP, the cGMP assay from GE Health was employed. 8,000 cells were seeded in 48-well plate for 24 hours. And cells incubated with peptides in PBS (without Magnesium and Calcium) containing 10mM of HEPES, 0.1 % FBS and

0.5 mM 1-methyl-3-isobutyl xanthine, which is an inhibitor of cyclic nucleotide phosphodiesterase, pH7.4 at 37° for 30minutes. The reaction was terminated by aspirating the reagents and the cells underwent lysis using *Lysis reagent* given in the cGMP kit. Finally, cells were tested in accordance with the manufacturer's protocol and the cGMP concentration was detected using the microplate reader.

3.2.8.2 Real time cell-viability

Cell viability was monitored using the Real-Time Cell Analyzer dual-plate (RTCA DP) Instrument, xCELLigence system (Roche Applied Science, Mannheim, Germany). The system measures the real-time cellular activities via electrical impedance detected from tissue culture E-plates that consist of integrated gold micro-electrodes at the bottom of the plates. The system measures the changes in impedance and converts them into a dimensionless parameter known as cell index (CI). The CI is calculated by the following equation $CI = \frac{(Z_i - Z_o)}{15}$, where Z_i and Z_o represent the individual point and background measurement at the commencement of the experiment respectively. The change in impedance occurs due to change in number of cells attachment or due to dimensional changes of attached cells.

First, 50 μ L of culture medium was added to determine the background of the E-plates. Next, 100 μ L of HCF cell suspension was added (8,000 cells/ well) and allowed to settle before the addition of another 50 μ L of culture medium. In the pre-conditioning step, cells were grown, conditioned in serum free medium and stimulated with CT-1 for 20-24 hours each sequentially. The impedance was monitored hourly during the entire preconditioning. For the daily dose treatment groups, the cells were

replenished with CD-NP daily. For film treatment groups, samples were prepared as 0.25 x 1 cm long strips that lined against the culture plate wall and fully immersed but not touching the cells. The impedance of both the daily dose and film treatment groups were monitored hourly, the CI values were calculated and plotted against time on graph. Additionally, relative cell index (RCI), a mean of comparing treatment group with respect to control group was calculated to be $RCI = \frac{CI_{Treatment}}{CI_{Control}}$, where $RCI < 1$ denotes inhibition of cell viability.

3.2.8.3 DNA synthesis

Replication of cellular DNA is an indication of cell proliferation; to measure the cellular DNA synthesis the Roche ELISA BrdU colorimetric assay was used. BrdU (5'-bromo-2'-deoxyuridine) is an analog of thymidine, which is one of the four DNA nucleobases. During mitosis, cells undergo DNA replication prior cell division. This assay works by incorporating colorimetrically-labelled BrdU label into newly synthesized cellular DNA, higher detection of absorbance indicates more cellular proliferation.

Treatment for HCFs

8,000 cells were seeded in 96-well plate, and cells were allowed to seed for 24 hours prior treatment. Cells underwent serum-deprivation for 24 hours to induce quiescent. Quiescent cells were incubated with stimulated with CT-1 for 24 hours. Next, CD-NP and CD-NP released from formulation were added into HCF.

Treatment for HCaSMCs

8,000 cells were seeded in 96-well plate and they were allowed to seed for 24 hours prior treatment. Cells underwent serum-deprivation for 24 hours to induce quiescent. Subsequently, the quiescent cells were incubated with CD-NP and CD-NP released from formulation for designate number of hours along with controls (without addition of peptide).

Post-treatment for HCF and HCaSMCs

Next, cells were labeled with the BrdU label for 24 hours. Finally, cells were fixed and tested in accordance with the manufacturer's protocol and measured at absorbance 370nm (Tecan microplate reader). Relative DNA syntheses were compared between control (no addition of CD-NP) and groups with addition of different concentrations of CD-NP. For CD-NP studies, "controls" refers to formulation without addition of CD-NP and "negative controls" refers to formulations without addition of CD-NP and FBS. For CD-NP film studies, "controls" refers to film formulation without addition of CD-NP and "negative controls" refers to film formulations without addition of CD-NP and FBS. The relative proliferative value of control groups were set as 1. All treatment groups were normalized to the respective controls.

Chapter 4 Formulation and film studies

4.1 Introduction

In chapter 2, we highlighted the therapeutic benefits of chimeric NPs. The physiological benefits and compatibility with the human body makes NPs ideal therapeutic agents. We also illustrated the challenges to realizing their therapeutic potential. These challenges could be summarized into the following:

- 1) NPs are *intrinsically hydrophilic*, which makes encapsulation in hydrophobic matrices, extremely difficult.
- 2) The *short biological half-life* implies that it has to be supplied regularly to achieve sustained therapeutic effect.
- 3) *Ideal release profile is unknown*; hence it is essential to elucidate the desired therapeutic levels and understand what type of release profile is optimal for tackling the pathology.

This chapter begins with tackling the first challenge, where the usage of co-solvent systems to overcome the issue of CD-NP hydrophilicity is investigated. In this chapter, we explored 2 types of co-solvent system—co-solvent system 1: ethanol/DCM and co-solvent system 2: water/DCM. The former co-solvent system is miscible while the latter co-solvent system is immiscible. The second challenge necessitated the release to be studied over an extended period of time; the study was carried out for 30 days to understand which profiles gave better sustained release over time. To overcome the

third challenge, we attempted to tailor the release profiles by adding excipients. The usage of excipients as blends is a simple, direct and efficient approach.

4.2 Co-solvent system 1: Ethanol/DCM

CD-NP is a predominantly hydrophilic peptide and it has very low solubility in DCM. In a preliminary experiment, CD-NP and PCL pellets were mixed together in single solvent DCM and casted into films. The films cast showed severe CD-NP aggregation on the surface. This prompted the introduction of co-solvent system, with the following criteria:

1. The two solvents chosen required one solvent to solubilize PCL pellets and the other solvent to solubilize CD-NP.
2. Formulations must be able to form films and they should not be brittle as they need to be used as device or coatings in biomedical applications.
3. Peptide release profile is appropriate with respect to the intended medical application.
4. The biological and physiological functions of CD-NP must be retained.

Ethanol/DCM and water/DCM co-solvent systems chosen were able to fulfill criterion 1 and 2 and they were further investigated. In sections 4.2 and 4.3, the two systems were studied to understand if they could meet criteria 3. Readers are referred to chapter 5 and 6 for studies pertaining to criteria 4. In this section, co-solvent system 1 consisting of ethanol and DCM is described.

4.2.1 In vitro release

Figure 4.1 shows the accumulative release profiles of CD-NP from films. The release profiles of CD-NP from neat PCL and PCL films with the addition of excipients were investigated. The results show that the release of CD-NP from neat PCL had the lowest initial release of 33 % on day 1 and final release of 97 % by the end of 30 days. In Figure 4.1a, an increase in initial release of 40 % and 55 % from formulations with addition of 5 % and 10% PEG was observed on day 1 respectively. And subsequently, formulations with addition of PEG continued to release to 96-97 % by 30 days. The formulations with addition of copolymer also had more pronounced initial and final release amounts compared to PCL. And in Figure 4.1b, an increase in initial release of 47 % and 73 % from formulations with addition of 5% and 10% copolymer was observed on day 1 respectively. Formulations with addition of copolymer yielded similar final release compared to PCL, where they release 95-96 % by 30 days.

The addition of PEG and copolymer appears to have resulted in increase in the initial release. The increment in initial release appears to be associated to amount of excipients added. We hypothesize that the faster initial release could be attributed to:

1. PEG and/or copolymer inducing pore formations when they leach out
2. CD-NP leaching out along with the excipients

Figure 4-1 Accumulated release profiles of 1% CD-NP loaded films with and without addition of PEG and copolymer PCL (1.5k)-PEG (5k). Accumulated release of a) PCL films with and without addition of PEG and b) PCL films with and without addition of copolymer

4.2.2 In vitro degradation

To characterize the in vitro degradation, the change in molecular mass and total film mass was studied. In figure 4.2 a, the molecular mass study carried out over 30 days showed no significant molecular mass change for both the pure PCL and excipient added formulations. In figure 4.2 b, the percentage mass loss recorded for PCL was 2.44 ± 0.05 % by 30 days. The films with addition of PEG displayed higher mass loss of 4.68 ± 0.43 % and 10.10 ± 0.30 % for 5 % PEG addition and 10 % PEG addition respectively. Similar mass loss trends were observed in films with addition of copolymers, where 5 % and 10 % copolymer addition experienced 3.97 ± 0.49 % and 7.91 ± 0.41 % mass loss respectively. Greater mass loss appeared to coincide with higher amounts of PEG and copolymer added to the films.

Figure 4-2 (a) Molecular mass change and (b) mass loss of CD-NP loaded films with and without addition of PEG and copolymer PCL (1.5k)-PEG (5k) over 30days

4.2.3 Surface morphology

The surface morphology of formulations before and after 30 days immersion in PBS was investigated. Figure 4.3 shows the surface morphology of films at 0 day and 30 days time point. From the SEM micrograph of PCL films loaded with CD-NP, no surface aggregation of CD-NP was observed. The image of PCL film after 30 days showed no degradative features. From the SEM micrograph of films with addition of PEG, the surface appeared slightly porous. The morphology of 5 % and 10 % PEG addition films looked similar. The surface morphology of films with addition of 5 % copolymer displayed “dimpled” surface, while films with addition of 10 % copolymer had small pores on the surface. All films with addition of PEG and copolymer showed no degradation by 30 days.

From the results, both the addition of PEG and copolymer displayed slight morphological changes in terms of emergence of “dimpled surface” and pore formation. After 30 days immersion, all films maintained their integrity. The absence of degradation in films was further verified by the molecular mass data (refer to section 4.2.2).

Figure 4-3SEM micrograph of 1% CD-NP loaded PCL film, addition of PEG film and addition of copolymer PCL(1.5k)-PEG (5k) on day 0 and after day 30 release

4.2.4 Mathematical analysis

To compare the peptide release profiles, a model-independent pair-wise comparison developed by Moore and Flanner was used [197]. This method calculates 2 factors, the difference factor and the similarity factor.

The difference factor, f_1 , is used to measure the percent error between two release profile curves over time. The formula is given as follows:

$$f_1 = \frac{\sum_{j=1}^n |R_j - T_j|}{\sum_{j=1}^n R_j} \times 100$$

Where n is the sampling number, the R_j and T_j terms are the percent released drug of curve 1 and curve 2 at each time point j . The percent error is zero if curve 1 and curve 2 profiles are identical and as the value increases it indicates greater degree of dissimilarity between profiles.

The similarity factor, f_2 , applies logarithmic transformation of sum squared error of differences between curve 1 and 2 over all time points.

$$f_2 = 50 \times \log \left\{ \left[1 + (1/n) \sum_{j=1}^n W_j |R_j - T_j|^2 \right]^{-0.5} \times 100 \right\}$$

where, w_j is the optional weight factor. The similarity factor has a range from 0 to 100. A similarity factor of 100 signifies that the profiles are identical and a similarity factor towards 0 signifies the increase in dissimilarity.

Although both f_1 and f_2 is presented, more emphasis is placed on f_2 as parameter of indication. Moreover, f_2 is widely used as indicator in literature and it is also endorsed by FDA as guidance for release from solid dosage forms. Furthermore, f_2 gives a definitive range, where f_2 of 50 and above signified that the profiles were similar and f_2 value below 50 considers the profiles as dissimilar.

Pair-wise comparison was performed to compare the PCL film against film with addition of excipients. Table 4.1 presents the f_1 and f_2 to indicate the differences observed between the release profiles.

We compared the similarity factor f_2 between PCL and formulations with 5 % PEG addition and 10 % PEG addition and found the f_2 to be 58.0 and 41.6 respectively; this denotes that 10 % addition of PEG significantly altered the release profile apart from increasing the initial release. When comparing the PCL formulation to the 5 % copolymer formulation, f_2 of 54.2 was obtained, denoting no difference in release rate. On the other hand, f_2 of 31.1 was obtained when PCL and 10 % copolymer formulation were compared, indicating that the release was significantly altered. Although, both the addition of 5 % and 10% excipient increased the initial CD-NP released, only 10% addition of excipient significantly altered the release profile.

Table 4-1 Similarity and different factor comparing formulations in ethanol/DCM system

Compared to PCL	Different Factor	Similar Factor
	f1	f2
5% PEG	4.7	58.0
10% PEG	29.7	41.6
5% Copolymer	14.2	54.3
10% Copolymer	22.9	31.1

4.3 Co-solvent system 2: Water/DCM

In this section, co-solvent system 2, consisting of water and DCM is described. Due to immiscibility nature of DCM and water, time-dependent solution turbidity was observed. When solution was prepared for short emulsification (SE, 10 minutes) the solution appeared clear and when the system underwent long emulsification (LE, 1 hour) the solution appeared turbid. Figure 4.4a shows a photograph of the polymer solutions of two emulsification conditions. Time-dependent solution turbidity in immiscible solvents is expected and matrix morphological changes had been reported in literature. However, in depth studies on the effects on drug distribution, degradation and release mechanism had not been reported. This section investigates the effects of solution emulsification condition on the properties and release kinetics. Apart from the emulsification duration, all parameters including the drying time, amount of chemicals and thickness were kept constant. Furthermore, the addition of PEG and copolymer were subsequently investigated.

4.3.1 In vitro release

Figure 4.4 b shows the accumulated release profiles of CD-NP from PCL under SE and LE conditions. The release from the LE showed a huge initial release of 62 % in the 6 hours time-point whilst the release from the SE showed an initial release of 12 %, which is approximately a 5-fold difference. Subsequently, the release from the LE showed near complete release of 97 % while the release from SE attained 72 % release by 30 days.

Figure 4-4 a) Image of polymer-peptide solution stirred under short emulsification (SE) and long emulsification (LE) and b) Accumulated peptide release comparing the effects of SE and LE duration in Pure PCL

It appears that the difference in emulsification duration did result in drastic release difference. We suspected that this difference between the SE and LE films are due to:

1. Difference in film porosity.
2. Difference in peptide distribution within films

4.3.2 Effects of excipients on in vitro release

Next, the addition of PEG and copolymer were investigated and regardless of emulsification duration, the degree of solution turbidity was not visually distinctive. Figure 4.5 shows the release of CD-NP from PCL and formulations with 5 % and 10 % PEG addition. From figure 4.5 a, the release from SE films showed that the addition of PEG resulted in increase in initial release compared to PCL. PCL had 12 % initial release, while films with 5 % and 10 % PEG addition observed an initial release of 24 % and 27 % respectively. Films with PEG addition showed slightly higher final release of 75 % and 78 % for 5 % and 10 % PEG addition respectively. From figure 4.5 b, the release from the LE PCL and formulations with 5 % and 10 % PEG addition is shown. The release from LE films showed that the addition of PEG resulted in decrease in initial release compared to PCL. PCL had 62 % initial release, while films with 5 % and 10 % PEG addition observed an initial release of 24 % and 40 % respectively. However, all 3 films attained eventual release between 95-97 % by 30 days.

Figure 4-5 Accumulated CD-NP release from films with and without addition of PEG manufactured under a) short emulsion (SE) and b) long emulsion (LE) conditions

Figure 4.6 shows the release of CD-NP from formulations with 5 % and 10 % copolymer addition. From figure 4.6 a, the release from SE films showed that the addition of copolymer resulted in increase in initial release compared to PCL. Both copolymer addition films observed higher initial release of 36 % and higher final release of 80 % and 83 % for 5 % and 10 % copolymer addition respectively. Figure 4.6 b shows the release from the LE formulations and the addition of copolymer decreased the initial release compared to PCL. LE PCL observed 62 % initial release, while 5 % and 10 % copolymer addition formulations observed 31 % and 36 % initial release respectively. All 3 films attained final release between 96-97 %.

Figure 4-6 Accumulated CD-NP release from films with and without addition of copolymer manufactured under a) short emulsion (SE) and b) long emulsion (LE) conditions

Evidently, the addition of PEG and copolymer under the different emulsification duration resulted in different trends, where excipient addition resulted in increase in initial release in the SE and decrease in initial release in the LE. We suspect that the different trends observed are due to:

1. Changes in the distribution of CD-NP within films due to the addition of excipients.
2. Changes in film structures due to the addition of excipients

4.3.3 In vitro degradation

The degradation study was investigated by measuring the molecular mass change in the films. Figure 4.7 a and b shows the molecular mass changes of SE and LE films respectively over 30 days. All films from both SE and LE did not experience significant molecular mass changes, denoting lack of polymer degradation.

Figure 4-7 Molar mass change of CD-NP loaded films with and without addition of PEG and copolymer manufactured under a) short emulsion (SE) and b) long emulsion (LE) conditions.

4.3.4 Surface morphology

The surface morphology of formulations before and after 30 days immersion in PBS was investigated. Figure 4.8 shows the initial and post-30 days morphology of PCL films manufactured under the SE and LE conditions. The PCL films formed by SE and LE displayed very distinctive surface morphology, where the SE films had small pore formed between spherulites of PCL whilst the LE films had huge “crater-lake” structures formed. Figure 4.9 shows the initial and post-30 days morphology of films with addition of 5 % PEG (figure 4.9 a) and 10 % PEG (figure 4.9 b) addition

manufactured under the SE and LE conditions. 5% PEG addition films had “dimpled” surface for SE formed films and small pores formed for LE formed films. On the other hand, 10 % PEG addition films had “push-pin” surface for SE formed films and small pores formed around spherulites for LE formed films. It appears that the formation of pores is more distinct in LE formed films regardless of the amount of PEG added.

Figure 4.10 shows the initial and post-30 days morphology of films with addition of 5 % copolymer (figure 4.10 a) and 10 % copolymer (figure 4.10 b) addition manufactured under the SE and LE conditions. 5 % copolymer addition films had “dimpled” surface for both SE and LE formed films. On the other hand, 10 % copolymer addition films showed small pores formed on the surface of both SE and LE formed films. It appears that the surface morphology was independent of the emulsification duration for films with addition of copolymers. From figure 4.8, 4.9 and 4.10, surface morphology and integrity of all formulations after 30 days were maintained.

Figure 4-8 SEM micrograph of CD-NP loaded PCL film manufactured under a) short emulsion (SE) and b) long emulsion(LE) conditions on day 0 and after day 30 release

Figure 4-9 SEM micrograph of CD-NP loaded PCL film with addition of PEG manufactured under a) short emulsion (SE) and b) long emulsion (LE) conditions on day 0 and after day 30 release

Figure 4-10 SEM micrograph of CD-NP loaded PCL film with addition of copolymer manufactured under a) short emulsion (SE) and b) long emulsion (LE) conditions on day 0 and after day 30 release

4.3.5 Peptide distribution

To understand the peptide distribution within the films, the CLSM was used to monitor the distribution of peptide, PEG and copolymer in the films. Figure 4.11 displayed the CLSM images of films in respective windows of 514 nm excitation, 405 nm excitation, bright field and combine of all three components. Each image is a compilation of 39 μm thickness. For the SE and LE PCL formulations, there was no detection of fluorescence in the 514 nm excitation window, denoting the absence of PEG and copolymer. For the SE and LE 10 % addition PEG formulations, fluorescence was observed in both the 514 nm and the 405 nm excitation windows, denoting the presence of PEG and CD-NP. The fluorescence observed in the windows coincided in shape and position. Similarly, for the 10 % addition copolymer formulation, fluorescence was observed in both the 514 nm excitation window and the 405 nm excitation window, denoting the presence of copolymer and CD-NP. Furthermore, the fluorescence observed in both windows also coincided in shape and position. For both the addition of PEG and copolymer, the fluorescence sizes were larger in the LE compared to the SE process.

Figure 4.12 shows the CLSM images of CD-NP distribution in PCL film produced by SE and LE respectively. The fluorescence intensity throughout the SE film is very low, whilst the fluorescence intensity in the LE film was detected throughout the entire 39 μm thickness. From the CLSM images of films with addition of 10 % PEG, the SE film showed fluorescence distribution from 0 to 19.5 μm , whilst LE films showed fluorescence distribution throughout the 39 μm thickness. From the CLSM images of films with addition of 10 % copolymer, the SE film showed fluorescence distribution

from 0 to 32.5 μm thickness of films, whilst LE films showed fluorescence distribution throughout the 39 μm thickness.

Figure 4-11 CLSM image of CD-NP loaded PCL film, 10% addition of PEG film and 10% addition of copolymer PCL-PEG (1.5k-5k) manufactured via short emulsion(SE) and long emulsion(LE) conditions. Each window: 514 Ex, 405 Ex, bright field and Combine

Figure 4-12 CLSM image of CD-NP loaded PCL film, 10% addition of PEG film and 10% addition of copolymer PCL (1.5k)-PEG (5k) manufactured via short emulsion (SE) and long emulsion (LE) conditions. Each image represents the combine of 514 Ex, 405 Ex, each section is 6.5 μm with the images from left to right denoting the position of the film from top to bottom.

4.3.6 Mathematical analysis

In this section, we cross-compared CD-NP release profiles from the same formulation but manufactured under the 2 emulsification conditions. Figure 4.13 shows the comparison of same formulation release profiles under the SE and LE conditions.

Evidently, the addition of PEG and copolymer resulted in more similar release profiles between different emulsification condition groups compared to PCL. In this section, we use the similarity and different factors described in section 4.2.4 to compare profiles between different emulsification conditions. Readers are referred to section 4.2.4 for the explanation and equation of the pair-wise model.

PCL SE and LE release profiles were compared and the f_2 value is 19.6, denoting that the release profiles were very different. The f_2 value comparing SE and LE from formulations with addition of PEG were tabulated, addition of 5 % resulted in f_2 of 36.3 whilst the addition of 10 % PEG resulted in f_2 of 37.2. The addition of 5 % copolymer resulted in f_2 of 49.3 whilst the addition of 10 % PEG resulted in f_2 of 45.6. The results indicate that addition of copolymer resulted in more similar profiles. A summary of the f_1 and f_2 results is found in table 4.2.

A comparison between different excipient at same concentration was also compared. A summary of the f_1 and f_2 results is found in Table 4.3. The f_2 values comparing SE and LE from formulations with 5 % concentration of different excipient are 45.5 and 76.5 whilst the addition of 10% concentration of different excipient resulted in f_2 of 52.5 and 69.8 respectively. The results indicate that at 5 % addition of different excipients, SE resulted in different profiles while LE resulted in similar profiles. And 10 % addition of different excipient resulted in similar profile regardless of emulsification duration.

Figure 4-13 Accumulated peptide release comparing the effects of short emulsion (SE) and long emulsion (LE) duration in PCL formulation, addition of 5% PEG, addition of 10% PEG, addition of 5% copolymer and addition of 10% copolymer formulations

Table 4-2 Different and similar factor comparing the release profiles between short emulsification and long emulsification of PCL and formulations with addition of PEG and copolymer in water/DCM system

	Different Factor	Similar Factor
Formulations	f1	f2
PCL	98.9	19.6
5% PEG	42.6	36.3
10% PEG	43.5	37.2
5% Copolymer	21.3	49.3
10% Copolymer	28.1	45.6

Table 4-3 Different and similar factor comparing the release profiles of different excipient at same concentration in water/DCM system

Compare PEG and copolymer formulations	Different Factor	Similar Factor
	f1	f2
5 % Excipient-SE	29.5	45.4
5 % Excipient-LE	4.8	76.5
10 % Excipient-SE	21.5	52.5
10 % Excipient-LE	6.1	69.8

4.4 Discussion

Miscible co-solvent system 1: Ethanol/DCM

In this chapter, two systems used in the manufacturing of films for the entrapment of CD-NP were described. First, the ethanol/DCM co-solvent system was explored. In

neat PCL films loaded with CD-NP, we achieved sustained release till 30 days. PCL films displayed a small initial release followed by subsequent release and finally releasing 97 % of the drugs by 30 days. As there was an absence of polymer degradation observed from the degradation study, we believe that the release is likely attributed to dissolution of peptide near the surface of the films and diffusion of the peptide from the matrix.

In this work, the effects of excipient addition were also investigated. The rationale for adding excipient is to provide more tailoring of the release profile and understand the effects of such addition on film properties and peptide release. We started by investigating the effects of addition of 5 % and 10 % PEG to the films. The addition of hydrophilic excipient PEG resulted in increase in the initial release. The addition of 5 % PEG increased the initial release by 1.2-fold whilst the addition of 10 % PEG increased the initial release by 1.7-fold. Further analysis on the release profile showed that 10 % PEG addition resulted in significantly different profile compared to PCL ($t_2 = 41.62$). This observation could be attributed to the physical and chemical changes upon the addition of PEG [159, 161, 198-200]. From the SEM micrograph of films with PEG addition, there were observable pores on the surface of films. This suggests that there is formation of micro-cavities within the films, which provided alternative pathways for the peptides to exit. From the mass loss data, we observed that addition of PEG resulted in increase in initial mass loss. This implies that mass lost is possibly due to the leaching of the excipient. We believe that the higher initial peptide release is associated with the peptides leaching out along with the excipients. Liu et al had also reported on the co-localization of protein and hydrophilic excipients, resulting in their simultaneous exit from polymeric films [198].

Next, we investigated an amphiphilic excipient which is copolymer PCL-PEG. The addition of amphiphilic copolymer was meant to facilitate better encapsulation of CD-NP, where the hydrophilic segment interacts with the peptide while the hydrophobic segment amasses with the rest of the PCL system [199, 201]. The addition of 5 % copolymer resulted in increase of initial release by 1.4-fold, while the addition of 10 % PEG increased the initial release by 2.2-fold. The 10 % copolymer addition resulted in higher initial release than the PEG formulations which was beyond our expectations. We rationalized that immediate leaching of the PEG segment in the higher copolymer concentration formulation probably resulted in the rapid release. The difference in the 5 % and 10 % copolymer addition also implied that copolymer addition amount is critical in producing significant phase separation which ultimately led to the eventual release profile. Previous works also demonstrated a similar effect when copolymers were added although the effects were less drastic due to the usage of high molecular mass PEG used [199]. Furthermore, copolymer appears to affect the initial release in a more dramatic manner. This is particularly so when we compare the 10 % copolymer addition formulation to the 10 % PEG addition formulation. The higher initial release might be a resultant of the higher molecular mass of PEG (5k) found in the copolymer compared to the PEG (2k).

In this co-solvent system, all formulations were able to sustain release over 30 days. The addition of excipients as a mean to modify peptide release has shown to be an effective strategy. The amounts of excipients added affects the release behavior and type of excipient added also results in different initial release. The initial peptide

release seems to correlate with the formation of micro cavities and the leaching of the excipients.

Immiscible co-solvent system 2: Water/DCM

We investigated the co-solvent system consisting of DCM-based polymer solution and water-based peptide solution. In the immiscible co-solvent systems, the emulsification duration changes the polymer-peptide dynamics and the eventual underlying peptide release mechanism. Despite the prevalence of transition of turbidity in polymer solution during emulsification, the influence of time factor has not been investigated in depth. In fact, the desirability of such turbidity had been debated. On one hand, such transition in turbidity has been used as means to provide tailoring of profile and matrix variability [172, 202]. On the other hand, some claimed that polymeric solution instability is catastrophic to the bioactivity of proteins and peptides [203, 204].

Films manufactured from 2 emulsification conditions were investigated on the peptide release, physical properties of films and distribution of peptide in the films. In neat PCL formulation, the emulsification duration significantly altered the initial release, where peptide release from the short emulsification (SE) films was 5-fold lower than long emulsification (LE) films. From the in vitro degradation studies, there was no significant degradation by 30 days. It was expected as PCL is a slow degrading polymer [140, 205]. Hence, it is evident that the release kinetics is independent on polymer degradation. So what contributes to this significant difference?

Effects of emulsification

Upon investigating the morphology, SE films had small cavities formed between PCL spherulites but larger pores were found in LE films. The large pore formation could be traced back to the film formation process, which is described in three stages—Solution state, semi-solution and semi-solid state. In the solution state, the hydrophilic domain formed droplets consisting water and CD-NP coalesce. Under long emulsification, hydrophilic domains have ample time to coalesce. Upon casting, drying occurs and the semi-solution state commence, where the hydrophilic domains move towards the surface facing the air. And in the semi-solid state, preferential early and faster loss of the more volatile solvent from the system [206] cause DCM to evaporate first leaving behind slow evaporating domains to eventually form “crater lake” structures. The “crater lake” pores house large surface areas of desorbed peptides and probably facilitated the migration of the peptide to the surface of the film, where they desorbed rapidly upon immersion in buffer. Further investigation carried out using the CLSM showed that SE films had no aggregations, suggesting that peptide is homogenously dispersed throughout the film. On the other hand, we discovered huge peptide aggregates in the LE films. The observed aggregation in LE is expected because hydrophilic domains had more growth time during the solution state and at the semi-solution state aggregation is likely to spread because the solution shifts from 3 dimensional to 2 dimensional geometry. Porous structure enlargement and peptide aggregations contributed to higher initial release observed in LE films.

Effects of excipient

The addition of excipients to formulations was considered in two parts—SE and LE condition. In the first part, we investigated the effects of excipients addition in SE condition. The addition of PEG and copolymer increased the initial release. This was

expected because from literature, the addition of excipients in polymer systems, often result in amplified release [131, 172]. And most groups would attribute it to loosely bound peptide at the surface [143, 165, 166, 173] or cavity formation as a resultant of excipient leaching [13, 76, 160, 164, 167-169]. From our investigation, the addition of excipients resulted in “dimpled” surface structures suggesting the possibility of micro-cavity formation within the matrix. From the CLSM data of formulations with excipients, we observed emergence of peptide-excipient co-localized aggregates. The increase in porosity and the presence of aggregates explains why excipients acted as accelerator of CD-NP release.

In the second part, we investigated the effects of excipient addition in LE condition. Under the LE condition, the addition of excipients resulted in decrease in initial release. There are few reports on PEG decreasing initial release [165, 166]. Some authors attributed the observation to altered solubility or reversible precipitation of drugs in the presences of excipients. It should be noted that in these studies, the addition of excipients eventually led to higher release compared to formulations without excipient addition. From the CLSM, films with and without addition appeared to have similar peptide distribution. From our morphology study, the addition of excipients resulted in disappearance of “crater” structures observed in neat PCL. Instead, the formation of micro-pores was observed on the surface of the excipient addition films. These observations imply that the reduction of pore size decreased the surface areas housing the peptides which decreased the initial release.

Effects of excipient and emulsification

When we cross-compared the SE and LE excipients-formulations profiles to the neat PCL, the release profiles appeared to be converging. This observation was further verified by the similarity factor analysis where formulations with excipient addition were deemed to be more similar, particularly the 10 % copolymer addition formulation. This suggest that despite conflicting trends observed, the roles of excipients should not be misunderstood as different under the two emulsification conditions. To understand the roles of PEG and copolymer under the different emulsification conditions, we compared the peptide distribution within the films. From the CLSM data, it appears that PEG co-localized with CD-NP to form hydrophilic rich domains within the matrix. PEG formulations manufacture via the SE condition had peptide-PEG domains distributed at 33-50% of film thickness whilst LE condition had peptide-PEG distributed throughout the films. The peptide distribution of copolymer addition formulations were investigated and found to play similar roles as PEG in terms of CD-NP distribution in films. Although, copolymer co-localized with CD-NP within the matrix in a similar fashion to PEG, the SE 10% addition copolymer films had peptide distributed about 66-83% of the films thickness whilst LE copolymer films had peptide distributed throughout the entire thickness of the film. The more similar distribution throughout the thickness of films is probably a key reason for the similar release profiles observed. In the next section, we would discuss the roles the excipients played in respective systems.

Roles of PEG and copolymer

The usage of excipients in protein and peptide release has been incorporated for various reasons, which includes improving the peptide bioactivity [129, 159-161], enhancing matrix properties [162, 163] and assisting complete release [164-168].

Besides the aforementioned advantages, the addition of excipient as blend is a simple and direct means to manipulate peptide release. PEG is hydrophilic, non-toxic and widely used as blends to regulate drug release in polymeric matrices [13, 76, 116, 123, 129, 160, 162-171]. Apart from improving hydrophilicity, PEG had been reported to accelerate release via its pore generation capabilities.

In the sections mentioned earlier, we presented how the addition of PEG and copolymer affected the peptide release in a miscible co-solvent system and an immiscible co-solvent system. It appears that excipients played different roles in different systems.

Miscible co-solvent system

In the miscible co-solvent system, the usage of excipients as blends is straight forward. The addition of excipients serves two major purposes in the ethanol/DCM system. Firstly, the addition of excipients resulted in increase in the initial and final release. This observation could be attributed to the physical and chemical changes upon the addition of excipients [159, 161, 198-200]. The SEM micrograph of films with excipient addition had observable “dimpled surface” or pores formed on the surface of films, suggesting that the formation of micro-cavities within the film, which provides alternative pathways for the peptides to exit.

Secondly, variability of profiles could be tailored and controlled based on amount of excipients added. The peptide release is believed to be associated to the peptides leaching out along with the excipients. The correlation between the mass loss and initial peptide release further supports this postulation. Liu et al had also reported on

co-localisation of protein and hydrophilic excipients, resulting in their simultaneous exit from polymeric films [198].

Immiscible co-solvent system

Unlike miscible co-solvent system, immiscible co-solvent system embroils a time-dependent factor. Firstly, different trends were observed from films manufactured using the polymer solution that underwent different emulsification conditions. When excipients were added under SE condition, there was an increase in the initial release, which is in agreement with literature that PEG accelerates release [13, 76, 160, 164, 167-169]. However, when excipients were added under the LE condition, there was a decrease in the initial release. The observed disparity in excipient addition in the same system under different emulsification condition had never been reported.

Secondly, similar release was observed in formulations with addition of excipients despite the different emulsification conditions. This suggests that the addition of excipient acted as release kinetic stabilizer under varied emulsification. In fact, from the analysis of similarity factors, the copolymer addition had greater similarity between release profile manufacture from different emulsification duration. In the practical sense, this implies that for applications involving immiscible co-solvent system, the addition of copolymers with appropriate chain lengths and hydrophilicity as emulsion stabilizer is a viable option to ensure consistency in therapeutic agent release profiles.

Evaluation (application)

The choice of system to use is depended on:

1. Type of release profile needed

The therapeutic dose is a critical parameter in determining an efficacious or adverse outcome. With the complicity of the pathological events, it is important to understand the desired release profiles.

In cardiac remodeling, early phase remodeling occurs within the first 72 hours and dominantly involves the expansion of the infarct zone [6]. Whereas for post-stenting, rapid proliferation of HCaSMCs occurs in the first 14 days followed by continues proliferation until 28 days [9, 35, 36]. To effectively counter early phase ventricular remodeling, a short 3 to 5 days release profile might be satisfactory, whilst sustained release of 28 days profile is required for stents application. Understanding the pathology gives us a good indication of the required dosing duration.

2. Application

For the application of coated stents or fully biodegradable stents, uniform and smooth surface coating is essential because the exposure of stent struts post angioplasty surgeries will cause inflammation. And such inflammation may leads to patients suffering delayed healing and in-stent restenosis (ISR) [8-10]. Under such circumstances where surface coating need to be smooth and coating must not risk delamination, the formulations from the miscible system may be preferred.

3. Bioactivity

In a peptide delivery system, apart from obtaining targeted release, the retention of the bioactivity of the peptide is paramount. In fact, the loss of biological functions due to

manufacturing process had been the major issue for the encapsulation of proteins and peptides [207, 208]. Readers are referred to chapters 5 and 6 for results on bioactivity of released peptide.

Chapter 5 In vitro studies on human cardiac fibroblast cells (HCFs) inhibition

5.1 Introduction

In chapter 2, we illustrated how compensatory mechanism of cardiac remodeling eventually led to the loss of contractile function. Fibrosis is a distinct pathologic feature of LV remodelling, where the accumulation of collagen impairs ventricular contraction and relaxation [1, 2, 4, 7, 209]. Since cardiac fibroblast is responsible for the production of extra-cellular matrix (ECM) proteins, it is apparent that inhibiting the fibroblast cells would prevent collagen accumulation and fibrosis [209, 210].

In this chapter, we studied the effects of CD-NP on the inhibition of CT-1 induced HCF. After elucidating the effective dose and the extent of inhibition, we studied the effects of released CD-NP on HCF. From the release profiles that we studied in chapter 4, we proceed to select 3 profiles—fast, moderate and slow initial release. Here, the purpose is to understand if the release profile or kinetic impacted the inhibition of the HCF.

5.2 Effects of CD-NP on HCFs

5.2.1 Elevation of cGMP production due to CD-NP

cGMP is a second messenger produced when NPs bind to the NP receptors. Hence, the detection of intracellular cGMP when peptide is incubated with cells implies that the NP had bound to the receptor and converted GTP to cGMP. In literature, NP concentrations that have resulted in successful elevation of cGMP were between 10^{-10} M to 10^{-5} M. Hence, we incubated CD-NP of concentration $0.00037 \mu\text{g/mL}$ (10^{-10} M) to $37 \mu\text{g/mL}$ (10^{-5} M) with the HCF.

In figure 5.1, the addition of CD-NP to HCFs led to elevation in the cGMP level. The CD-NP concentration range of $0.0037 \mu\text{g/mL}$ (10^{-9} M) to $37 \mu\text{g/mL}$ (10^{-5} M) appeared to elicit elevation of cGMP significantly compared to control. Also, there appears to be a dose dependence relationship within the concentration range of $0.0037 \mu\text{g/mL}$ to $37 \mu\text{g/mL}$. The ANOVA test of significance was carried out between groups of 10-fold difference in concentration (figure 5.1 a) and they were not statistically different. When a comparison was done between concentrations of 100-fold difference (figure 5.1 b), statistical significance was observed.

It seems that chimeric peptide CD-NP is similar compared to the native NPs in cGMP elevation, particularly:

1. Dose-dependent elevation of cGMP due to concentration, where higher NP concentration observed greater production of intracellular cGMP.

2. The effective working range is between 10^{-9} M to 10^{-5} M.

Figure 5-1 Cyclic 3'5' guanosine monophosphate (cGMP) generation in HCF induced by a) 10-fold different and b) 100-fold different CD-NP concentration, * $p < 0.05$ versus control.

5.2.2 Effects of CD-NP on HCFs proliferation and viability

Next, we moved to understand the inhibitory effects of CD-NP on HCF. In particular, the CD-NP concentrations, duration and extent of inhibition were of interest. Two methods were employed to study the inhibition of HCF hypertrophy and hyperplasia—the BrdU proliferation assay and the xCELLigence system. Here, **HCF hyperplasia** refers to the multiplication of HCF cell number whilst **HCF hypertrophy** refers to the increase in the volume or size of HCF cells.

To understand the effects of CD-NP addition on cellular proliferation at the DNA level, the DNA synthesis in HCF were investigated. Figure 5.2 shows the relative

Chapter 5 In vitro studies on human cardiac fibroblast cells (HCFs) inhibition

DNA synthesis of HCF after the addition of CD-NP solution in concentrations of 37 $\mu\text{g/mL}$, 0.37 $\mu\text{g/mL}$ and 0.0037 $\mu\text{g/mL}$ for 24 hours. This gives us an indication on CD-NP's effect on HCF hyperplasia. All the tested CD-NP concentrations showed statistically significant suppression in the synthesis of DNA ($p < 0.05$), denoting suppression of HCF hyperplasia. However, no concentration dependence between CD-NP concentration and the amount of DNA synthesized was observed.

Next, we investigated the duration and extent of the inhibitory effect. CD-NP in concentrations of 37 $\mu\text{g/mL}$, 0.37 $\mu\text{g/mL}$ and 0.0037 $\mu\text{g/mL}$ were incubated with HCF for 2 days, 3 days and 5 days. Figure 5.3 shows the relative proliferation of CD-NP on HCF over 5 days. Although there appears to be lower DNA synthesis relative to control on the 2nd day, it was not statistically significant. All 3 concentrations showed suppression of DNA only on the first day. Single CD-NP dose suppressed DNA synthesis in HCF for up to 24 hours and does not extend beyond 24 hours.

Figure 5-2 Relative DNA synthesis of different CD-NP concentration in HCF for 24 hours via colormetric bromodeoxyuridine (BrdU), *p < 0.05

Figure 5-3 Relative DNA synthesis of different CD-NP concentration in HCF for 1,2,3 and 5 days via colormetric bromodeoxyuridine (BrdU), * $p < 0.05$

To understand how CD-NP affects the viability of hypertrophic HCF, the xCELLigence system was used to dynamically monitor HCF spread (hypertrophy) and proliferation (hyperplasia). Figure 5.4 shows the graphs of cell index (CI) of HCF against time, where CI increment denotes increase in cell proliferation or cell spreading. Prior to treatment with CD-NP, cells were seeded, medium-deprived and stimulated with CT-1 for one day each. All pre-treatments were carried out before 0 days and represented as negative days on the graph. After 24 hours of medium-deprived starvation, cells displayed unanimous decline in CI (day -2 to day -1). From

the results, we observe an increase in CI following the addition of CT-1(day -1 to day 0), indicating an increase in cell spread and/or proliferation. This observation is similar to studies on *in vitro* cultured cardiac fibroblast and *in vivo* animal studies, where CT-1 directly stimulates pathological hypertrophy and induces chamber dilation [6, 210, 211].

Single dose CD-NP at concentrations of 37 $\mu\text{g/mL}$, 0.37 $\mu\text{g/mL}$ and 0.0037 $\mu\text{g/mL}$ were administered at day 0. From figure 5.4, the CI profiles of all tested CD-NP concentrations appeared to be similar to the control. This indicates that single administration of CD-NP did not have observable effects on the viability of HCF.

Figure 5-4 Cell Index (CI) measurement of control compared to single dose infusion of different CD-NP concentrations in HCF at 0 day via the RTCA xCELLigence

5.2.3 Effects of multiple dose CD-NP on HCFs proliferation and viability

From 5.2.2, single dose of CD-NP inhibited DNA synthesis of HCF (hyperplasia) for 24 hours and lost inhibitory effects beyond 24 hours. When single dose of CD-NP was administered into HCF using the xCELLigence system (hypertrophic and hyperplasia), there were no visible inhibitory effects on HCF in terms of drop in CI. In view of the limited inhibition effects, this section attempts to study multiple dosing of CD-NP, in particular to investigate and understand the following:

1. Can multiple dosing sustain the inhibition of HCF cells?
2. Does multiple dosing have amplified effects on inhibition of HCF cells?

To address these queries, we proceeded to study the DNA synthesis of HCF with daily dose of CD-NP. The effects of daily dosing was investigated and shown in figure 5.5. DNA synthesis of HCF was observed to be suppressed in the presence of CD-NP. The suppression was found to be independent of concentration (37 $\mu\text{g/mL}$, 0.37 $\mu\text{g/mL}$ and 0.0037 $\mu\text{g/mL}$) for the first 3 days of daily infusion. However, only the highest concentration 37 $\mu\text{g/mL}$ retained effectiveness in inhibiting DNA synthesis in HCF on the 5th day.

Next, the effects of daily dose CD-NP on HCF cell viability was carried out using CD-NP concentration range between 0.0037 $\mu\text{g/mL}$ to 37 $\mu\text{g/mL}$. Figure 5.6 shows the CI

values of daily dose CD-NP at concentrations 0.0037 $\mu\text{g/mL}$, 0.37 $\mu\text{g/mL}$ and 37 $\mu\text{g/mL}$. CD-NP concentrations of 0.37 $\mu\text{g/mL}$ and 37 $\mu\text{g/mL}$ displayed eventual inhibition of hypertrophic HCF. Although, daily dose of CD-NP at 0.0037 $\mu\text{g/mL}$ also displayed decline in CI values compared to control, the difference was not statistically significant. This implies that multiple dose CD-NP is effective in inhibiting hypertrophic HCF.

Figure 5-5 Relative DNA synthesis of daily infusion of different CD-NP concentration in HCF for 1,2,3 and 5 days via colormetric bromodeoxyuridine (BrdU), * $p < 0.05$

Figure 5-6 Cell Index (CI) measurement of a) daily infusion of different CD-NP concentrations compared to control and b) 0.37 µg/mL daily dose CD-NP in HCF compared to control via the RTCA xCELLigence

5.2.4 Comparision of single dose and multiple doses CD-NP on HCFs

In sections 5.2.2 and 5.2.3, the results of single dose and multiple doses of CD-NP on HCF were presented. Figure 5.7 is the summary of the modes of release of single dose CD-NP and multiple doses CD-NP. The results suggest that multiple dosing of CD-NP is essential for sustaining the inhibition of DNA synthesis in HCF. Moreover, when high CD-NP concentration was given in multiple doses, more pronounced inhibition of HCF viability was observed.

The xCELLigence measures the impedance change based on number of cells attached and the dimensions of the attached cell; this implies that both HCF hyperplasia and HCF hypertrophy are measured. From the DNA synthesis study, single dose CD-NP inhibited HCF hyperplasia yet this inhibition was not observed in the xCELLigence study. This suggests that the drop in CI values when multiple doses CD-NP was administer maybe a resultant of inhibition of hypertrophic HCF. This would be discussed further in section 5.4.

Figure 5-7 (a) Mode of single dose CD-NP and (b) Mode of daily dose CD-NP

5.3 Effects of released CD-NP on HCFs

5.3.1 Review of selected release profiles

From section 5.2, we learnt that daily dosing is more effective than single dose in achieving sustained inhibitory effect on HCF. We hypothesize that a continuous supply of CD-NP via controlled release of CD-NP from polymeric films would give a sustained inhibitory effect equivalent to daily (multiple) dosing.

We proceed to select three distinct CD-NP release profiles to understand their effects on *in vitro* HCF. The selected profiles were chosen from the work reported in chapter 4. All films were loaded with 1% CD-NP and they displayed different release profiles due to their different co-solvent nature and varied emulsification time.

In this section we rename them as: Low, Moderate and High. The summary of formulation information is found in table 5.1.

Table 5-1 Formulations to be tested for HCF studies

	Initial	Co-solvent	Emulsification	CD-NP
Films	release	system	duration	loading
L	Low	Water/DCM	10 minutes	1 wt%
M	Moderate	Ethanol/DCM	10 minutes/1 hour	1 wt%
H	High	Water/DCM	1 hour	1 wt%

Before we proceeded to test the effectiveness of different release kinetics, the release percentage and concentration were reviewed. From figure 5.8 a, film L and film H had the lowest and highest initial release of 14 % and 65 % released at 6 hours respectively. Film M had a medial initial release of 31 % and attained 93 % release by the end of 30 days. Figure 5.8 b shows the concentration of the CD-NP released and the post initial release, all films fell in the range of 1-6 $\mu\text{g/mL}$ between 1 day and 30 days. For a detailed explanation on the release mechanism of each formulation, readers can refer to chapter 4.

Figure 5-8(a) Accumulated peptide release profiles of films L, M and H and (b) Release concentrations of films L, M and H over 30 days

5.3.2 Elevation of cGMP production

In section 5.2.1, the addition of CD-NP to HCF led to a dose dependent elevation of intracellular cGMP levels. To verify if CD-NP encapsulated in the films retained bioactivity, CD-NP released at the 24 hour time point from the films were measured. Figure 5.9 displays the cGMP production levels of the released CD-NP and all 3 formulations displayed statistically significant elevation of cGMP compared to the control group. The degree of bioactivity from encapsulated CD-NP could not be elucidated because only 100-fold difference in CD-NP concentration showed statistical significance in production of cGMP.

Figure 5-9 Cyclic 3'5' guanosine monophosphate (cGMP) generation in HCF induced by 24 hour CD-NP release from films L, M and H, * $p < 0.05$ versus control

5.3.3 Effects of released CD-NP on HCFs viability

In this section, xCELLigence results were expressed in CI and relative cell index (RCI). CI is the raw data obtained directly, while RCI is the normalized to the CI of control groups. RCI describes the cell viability in a comparative manner. RCI value less than 1 denotes inhibition of cells, whilst RCI more than 1 denotes otherwise.

Figure 5.10 shows the cell viability study of HCF of films L, M and H respectively against the control group. Both films L and H showed immediate decline of CI

compared to control, whereas film M showed decline in CI compared to control only on the 4th day. Figure 5.11 shows the correlation between the RCI (primary y-axis) and peptide concentration (secondary y-axis) with respect to time. From figure 5.11, we can see that daily dosing of CD-NP results in “spikes” delivery profile of CD-NP at 37 $\mu\text{g/mL}$ daily (secondary y-axis) but the RCI was only less than 1 after the 2nd day. Films L and H had RCI value less than 1 from 0 to 5 days. Film M however only showed RCI less than 1 after the 1st day. By the 5th day, all films had RCI less than 1.

Figure 5-10 Cell Index (CI) measurement of control compared to daily infusion of CD-NP, films L, M and H in HCF via the RTCA xCELLigence

Figure 5-11 Correlation between Relative Cell Index (RCI) (primary y-axis) and peptide concentration (secondary y-axis) of daily infusion of CD-NP, films L, M and H over 5 days (x-axis) in HCF

5.2.4 Effects of released CD-NP on HCFs proliferation

Figure 5.12 shows the effect of the 3 CD-NP releasing formulations on the relative DNA synthesis in HCF. All formulations showed statistically significant suppression of DNA synthesis for 2 days. Although all formulations appeared to show lower DNA synthesis on the 3rd day, it was not statistically significant. The absence of bioactivity by the 5th day from the released CD-NP was expected as the critical dose was not met.

Figure 5-12 Relative DNA synthesis of CD-NP released from films L , M and H (1 day, 2days, 3days and 5days) in HCF via colorimetric bromodeoxyuridine (BrdU), *p <0.05

5.4 Discussion

CD-NP on HCF

In the literature, CD-NP has been reported to exert anti-fibrotic actions and regulate homeostasis through the elevation of cGMP. In our study, pure CD-NP elicited elevation of cGMP production in a dose dependent manner similar to reports in literature. After verifying that CD-NP elicits cGMP production, we moved on to understand the inhibition effects of CD-NP on CT-1 induced HCF using two methods. First, the BrdU assay was used to measure the relative proliferation of HCF. To understand if CD-NP affected HCF hyperplasia, experiments to elucidate the amount

of DNA synthesis in HCF in presence of CD-NP were carried out. DNA synthesis of HCF in presence of CD-NP was suppressed but the inhibition was limited to 24 hours. Moreover, we found that the suppression was independent of the CD-NP concentration (37 $\mu\text{g/mL}$, 0.37 $\mu\text{g/mL}$ and 0.0037 $\mu\text{g/mL}$).

Next, the xCELLigence system was used to dynamically monitor HCF spread and proliferation. From the results, we observed that the CI of HCF treated with single dose CD-NP was similar to control. The results indicated that single dose CD-NP had no observable impact on HCF viability. The difference between the two experiments hinted that single dose CD-NP is more effective in inhibiting HCF hyperplasia than hypertrophy.

Single versus multiple dose

As single dose CD-NP had limited inhibitory capabilities, we wanted to understand if multiple dosing could amplify or sustain inhibitory effects. We proceed to study the effects of multiple doses CD-NP given on a daily basis on the inhibition of HCF hypertrophy and hyperplasia. In daily dosing, the suppression of DNA synthesis in HCF was found to be independent of dose (37 $\mu\text{g/mL}$, 0.37 $\mu\text{g/mL}$ and 0.0037 $\mu\text{g/mL}$) for the first 3 days of daily infusion. Subsequently, 0.37 $\mu\text{g/mL}$ and 0.0037 $\mu\text{g/mL}$ were rendered ineffective in the inhibition of DNA synthesis. However, for the highest concentration, 37 $\mu\text{g/mL}$, sustained inhibition was observed up to 5 days. This observation suggests that prolonged dose responsiveness differs from immediate dose responsiveness, which maybe associated to the interdependence of GC receptor pathways and NP exposure [124, 128]. Moreover, the suppression of DNA synthesis in HCF was sustained in the presence of CD-NP multiple dosing.

In the daily infusion of CD-NP group, the xCELLigence study showed that the commencement of inhibition started after the 3rd dose of CD-NP. This implies that multiple dosing is essential for effective inhibition of HCF. The xCELLigence study gave data pertaining to the hypertrophy and hyperplasia of HCF, whilst the DNA synthesis study gave indication on cell hyperplasia. Daily dosing of CD-NP suppressed DNA synthesis up to 5 days, yet this reduction in cell proliferation was not observed from the CI values of the xCELLigence experiment. This suggests that hypertrophic cells played a larger role of the manipulation of CI values as measured in the xCELLigence experiments. Subsequently, a decline in CI was observed after the third dose, which could be attributed to both suppression of cell hypertrophy or cell hyperplasia. It is therefore reasonable to conclude that, if we want to suppress both hyperplasia and hypertrophy of HCF, sustained (or multiple dosing) is essential.

Controlled release on HCF

Upon verifying and elucidating the extent of CD-NP's anti-fibrotic properties, the next step would be to test and understand the effects of sustained supply of CD-NP released from polymeric matrices on the HCF. We selected 3 formulations from our study in chapter 4, where 3 different initial release profiles in high, medium and low were selected. The retention of bioactivity of encapsulated CD-NP was first verified using the cGMP assay. CD-NP released from films showed elevation of cGMP, implying the retention of bioactivity in the processed films.

Next, the effects of sustained release were investigated by the xCELLigence study. Films L and H exhibited early and sustained inhibitory effects, which suggests that the

performance of a sustained supply of lower dose CD-NP surpasses that of a high-dose daily bolus infusion. This observation might be associated with the short elimination half-life of CD-NP (18.4 ± 1.4 minutes), where administered bolus only had short-lived biological effects [119]. Films L and H displayed an almost immediate and sustained inhibitory effect until 5 days. Film M has higher bursts and sustained release than film L but film L appeared more effective than film M. In film M, there was an absence of inhibition in the beginning and the inhibitory effects only became distinct on the 4th day. At present, the candidate does not have an explanation for this observation. However, the results seem to hint that CD-NP encapsulated in water/DCM system might have more superior bio-activity compared to the ethanol/DCM system. Such arguments had also been previously reported, where water is less harsh compared to organic solvents, provides hydration and does not implicate any toxicity issues. These properties make water an ideal co-solvent for the encapsulation of proteins and peptides [207].

Unintentional alteration resulting in loss of protein and peptide pharmacological functions is common [207, 208] but may be inevitable due to manufacturing processes involving polymeric matrices. We suspect that that the peptide encapsulated via the ethanol/DCM system had become less potently active compared to the peptide encapsulation prepared via water/DCM. However, in the cGMP elevation studies, we did not pick up any difference between CD-NP encapsulated in the water/DCM and ethanol/DCM systems. We have two explanations for this observation. Firstly, the difference in concentration of CD-NP needs to be at least 100 fold to be differentiated by the cGMP assay (refer to results). Hence, there is a need for a more sensitive

technique to resolve the discrepancy. Secondly, the detection of cGMP elevation may not be directly correlated to the mechanism of inhibition of proliferation.

Discrepancy of effects between pure peptide and released peptide

The suppression of DNA synthesis was observed from CD-NP releasing films. However, the suppression of DNA from films only lasted for the first 2 days. The absence of suppression by the 5th day from the released CD-NP was expected as the critical dose is not sustained. However, the lack of DNA suppression on the 3rd day was unexpected. As the CD-NP released was within the working range (0.0037 µg/mL to 37 µg/mL). We suspect that the lack of DNA synthesis suppression could be attributed to the different types of exogenous CD-NP profiles. The daily dosing of CD-NP has an “On-off” (bolus) profile (Figure 5.7), whilst film had an ‘on’ release profile throughout the entire duration. These different exogenous profiles elicit different cascading modes and biological responses; hence an “on-off” profile may elicit different DNA synthesis behaviour compared to the “on” profile.

Bioactivity and application impact

Loss of pharmacological functions due to the manufacturing process has been a major issue for encapsulation of proteins and peptides. It is crucial to verify that encapsulated CD-NP retained its bioactivity and the cGMP elevation was used for this purpose. Our study on the CD-NP released from film showed elevation of cGMP level, implying the retention of bioactivity.

As CD-NP is a relatively new therapeutic entity, the therapeutic window has not been fully established. From literature, the CD-NP infusion range between 0.1-30

ng/kg/minute (for 1-7 days) and 10-30 ng/kg/day (for 5-30 days) via intravenous and subcutaneous infusions were reported to be effective; however there is no literature on an ideal therapeutic profile [14]. This is also the main motivation for the development of films with different release profiles. The 3 films selected for further study achieved sustained release over time in the range of 12-84 $\mu\text{g/mL}$ (for 0-6 hours) and 1-6 $\mu\text{g/mL}$ (for 1-30days). Daily subcutaneous infusion of CD-NP at 10-30 ng/kg/day over period of 5 to 30 days had successful elevation of plasma and urine cGMP whilst improving cardiac load and minimizing arterial pressure [14]. Our films (1 cm $\times$ 1 cm $\times$ 0.004 cm) was able to achieve release of 1-6 ng/kg/day (between 1 to 30 days), suggesting that it could be potentially used as an alternative administrative route for treatment. Moreover, the thickness and the area of these films could be increased if a higher dose is required.

Current treatment options only serve to reinforce the LV mechanically but do not provide any functional benefits in preventing progression of disease or in restoring contractile functions of a failing heart. The implantation of CD-NP-releasing films subcutaneously or via device using minimally invasive procedures could be used to moderate fibrotic scar formation and ease cardiac remodeling. Furthermore, implantation of these biodegradable matrices or devices eliminates the need for a second surgery need for removal. However, in vivo studies need to be carried out to confirm and elucidate CD-NP's clinical effectiveness.

Chapter 6 In vitro studies on human coronary smooth muscle cells (HCaSMCs) inhibition

6.1 Introduction

As reviewed in chapter 2, the development of DES is an important milestone in the field of intervention cardiology. DES provides the mechanical means to keep the occluded vessel patent while minimizing the neointimal proliferation. However, the non-selectivity of the drug used in DES has resulted in delayed endothelialisation, giving rise to (sometimes fatal) late-stage thrombosis (LST) and also extended the duration for administration of anti-thrombotic drugs after stent implantation. Hence, the use of discriminatory therapeutics appears to be the key to resolving the issue. The peptide CNP has been reported to exhibit inhibition of HCaSMCs proliferation. Moreover, CNP is of endothelial origin, implying that it is compatible to endothelial cells. As a hybrid of CNP and DNP, CD-NP appears to be an ideal therapeutic to resolve these issues.

In this chapter we aim to elucidate the inhibitory effects of CD-NP on HCaSMCs and the effectiveness of released CD-NP. Since HCaSMC inhibition is the main factor in mitigating restenosis, we begin the work in this chapter by investigating the inhibition

effects of CD-NP on HCaSMCs. After elucidating the effective dose, duration and extent of inhibition, we studied the effects of released CD-NP on HCaSMCs. To understand if excipient addition enhanced or decreased the bioactivity of encapsulated CD-NP, we selected 3 formulations for investigation—pure PCL formulation, formulation with the addition of 10 % PEG and formulation with the addition of 10 % copolymer.

6.2 Effects of CD-NP on HCaSMCs

6.2.1 Elevation of cGMP production in HCaSMCs by CD-NP

First, we investigated if CD-NP binds to the NP receptors in HCaSMCs to elevates the production of intracellular cGMP in similar fashion like the native CNP. Figure 6.1 presents the effects of CD-NP on HCaSMCs production of cGMP. CD-NP in concentrations of 37 $\mu\text{g/mL}$, 0.37 $\mu\text{g/mL}$ and 0.0037 $\mu\text{g/mL}$ elevated the cGMP levels. Addition of concentrations of 37 $\mu\text{g/mL}$, the highest concentration studied, produced more than 2 times higher cGMP amounts compared to control. Also, there appears to be a dose-dependent relationship between concentration and cGMP levels. However, only 37 $\mu\text{g/mL}$ and 0.37 $\mu\text{g/mL}$ concentration were statistically significant compared to control ($P < 0.05$). CD-NP at concentration of 0.0037 $\mu\text{g/mL}$ did elicit cGMP production but it was not statistically significant compared to control.

Figure 6-1 Cyclic 3'5' guanosine monophosphate (cGMP) generation in HCaSMCs induced by CD-NP at different concentrations, * $p < 0.05$ versus control

6.2.2 Effects of CD-NP on HCaSMCs proliferation

Since the inhibition of HCaSMC is the main means to restrain restenosis, we examined the effects of CD-NP on inhibition of DNA synthesis in HCaSMCs. First, we investigated the inhibitory capabilities of CD-NP on HCaSMCs for 24 hours. CD-NP solution in concentrations of 37 µg/mL, 0.37 µg/mL and 0.0037 µg/mL were added to quiescent HCaSMCs and the proliferation rates were compared to the control as seen in figure 6.2. Although a decline in DNA synthesis was observed in the 37 µg/mL CD-NP group, the decline was not statistically significant.

Figure 6-2 Relative DNA synthesis of different CD-NP concentration in HCaSMCs for 24 hours via colormetric bromodeoxyuridine (BrdU), *p <0.05

Next, we investigated the inhibitory capabilities of CD-NP on HCaSMCs over the course of 7 days to understand if there was a delayed inhibitory effect. Figure 6.3 shows the relative proliferation of HCaSMCs after addition of CD-NP at day 1, day 3, day 5 and day 7. When compared to the control, there was no statistically significant inhibition.

Figure 6-3 Relative DNA synthesis of different CD-NP concentration in HCaSMCs for 1, 3, 5 and 7 days via colormetric bromodeoxyuridine (BrdU), *p < 0.05

The results obtained were unexpected because in literature strong inhibition had been reported in both CNP and CD-NP [108, 109, 212]. The discrepancy between results observed and literature prompted further investigation of experimental parameters, stimulant addition and interaction times which would be presented in sections 6.2.2.1 to 6.2.2.4.

6.2.2.1 Effects of cell seeding density and FBS

To understand if cell/media parameter differences affected inhibition effects of CD-NP, we investigated the effects of cell seeding density and percentage FBS. Cell

seeding density was investigated because of two reasons. Firstly, in the literature cell seeding density had not been reported in exact numbers and only in percentage cell confluence. Secondly, the density of cells is a crucial factor in the signaling, growth and proliferation of cells.

In section 6.2.2, HCaSMCs cell seeding density of 8,000 cells/well was used. We chose this seeding density because it was able to achieve 70-80 % confluence after 24 hours seeding. In this section, we investigated cell seeding densities of 4,000 cells/well and 12,000 cells/well, to see if reduction or increment of cells impacted the inhibitory activities of CD-NP on HCaSMCs. Figure 6.4 a and b shows the effect of CD-NP on HCaSMCs proliferation at densities of 4,000 cells/well and 12,000 cells/well respectively. The incubation of CD-NP for 24 hours at 4,000cells/well and 12,000cells/well did not have any statistically significant inhibition.

Next, the effect of FBS amount was investigated. In literature, cell proliferation activities were reported to be serum dependent [110] and the serum concentration varies in different pathological state [213-215]. The amount FBS recommended for normal growth of HCaSMCs was 5 % FBS, which was also the amount of FBS used in our results presented in section 6.2.2. In this section, we investigated FBS of 0 %, 1 %, 5 % and 10 %, to see if increased or decreased FBS impacted the inhibitory activities of CD-NP on HCaSMCs. Figure 6.5 shows the effects of CD-NP on cells grown in different FBS amounts. Despite the variation of percentage FBS amounts, interaction with CD-NP for 24 hours did not have any statistically significant inhibition on HCaSMCs.

Chapter 6 In vitro studies on human coronary smooth muscle cells (HCaSMCs) inhibition

Figure 6-4 Relative DNA synthesis of different CD-NP concentration in HCaSMCs for 24 hours via colormetric bromodeoxyuridine (BrdU) in a) 4,000 cells/well and b) 12,000 cells/well seeding densities, *p < 0.05

Figure 6-5 Relative DNA synthesis of different CD-NP concentration in HCaSMCs for 24 hours via colormetric bromodeoxyuridine (BrdU) under different % FBS conditions, *p < 0.05

6.2.2.2 Effects of ET-1 stimulation

Next, we moved to understand the effects of CD-NP on stimulated HCaSMCs. In literature, most groups induced the proliferation of HCaSMCs via nutrients deprivation followed by providing nutrient. HCaSMCs do not undergo hypertrophy under the influence of stimulant. Endothelin-1 (ET-1) had been reported to promote vasoconstriction and HCaSMCs proliferation [216].

In our procedure, cells were seeded; medium deprived and conditioned with ET-1(10^{-8} M) for 24 hours prior the addition of CD-NP. Figure 6.6 shows the effects of CD-NP on ET-1 stimulated HCaSMCs. The incubation of CD-NP with ET-1 stimulated HCaSMCs for 24 hours did not have any statistically significant effects. ET-1 conditioning was also evaluated at 12 and 36 hours, however the results were similar to conditioning HCaSMCs with ET-1 for 24 hours. (Results not shown)

Figure 6-6 Relative DNA synthesis of different CD-NP concentration in HCaSMCs for 24 hours via colormetric bromodeoxyuridine (BrdU) where SMCs were pre-treated with ET-1, *p <0.05

6.2.2.3 Effects of time-dependent peptide-HCaSMCs interaction

In this section, we investigate duration of CD-NP and HCaSMCs interaction time: 4 hours, 8 hours, 12 hours and 24 hours. Figure 6.7 presents the relative DNA synthesis of HCaSMCs upon interaction with CD-NP at 37 µg/mL for 4, 8, 12 and 24 hours. A decline in DNA synthesis was observed for the 4, 8 and 12 hour interaction time. The ANOVA test of significance was carried out and only interaction at 4 hours was found to be statistically significant.

Subsequently, a dose range study was carried out to elucidate the effective concentration at 4 hours. Figure 6.8 shows that CD-NP suppressed cell proliferation in HCaSMC between concentration of 0.0037 $\mu\text{g/mL}$ and 3.7 $\mu\text{g/mL}$ but beyond 0.0000037 $\mu\text{g/mL}$ inhibition was ineffective.

Figure 6-7 Relative DNA synthesis of CD-NP in HCaSMCs for 4, 8, 12 and 24 hours via colormetric bromodeoxyuridine (BrdU), * $p < 0.05$

Figure 6-8 Relative DNA synthesis of different CD-NP concentration in HCaSMCs for 4 hours via colormetric bromodeoxyuridine(BrdU), *p <0.05

6.2.2.4 Effects of temperature

From sections 6.2.2.1 to 6.2.2.3, we gathered that CD-NP exerts inhibition on in vitro HCaSMCs effectively for only 4 hours. So, why does inhibition only last for 4 hours? It appears that the reasonable explanation would be either that CD-NP loses its inhibitory activity beyond 4 hours of incubation or that the inhibitory effects of CD-NP are not sustained beyond 4 hours.

Chapter 6 *In vitro* studies on human coronary smooth muscle cells (HCaSMCs) inhibition

In this section, we first investigate the first postulation. We incubated CD-NP (in SmB medium) in 37 °C for 24 hours prior testing. Next, freshly prepared CD-NP and CD-NP incubated for 24 hour were allowed to interact with HCaSMCs for 4 hours. Figure 6.9 shows the relative proliferation of the two groups.

There is no significant difference between the inhibition effects of freshly prepared CD-NP and CD-NP prepared 24 h in advance for all concentrations. This signifies that CD-NP did not lose its inhibition capability due to incubation in 37 °C in SmB medium.

Figure 6-9 Relative DNA synthesis of freshly prepared CD-NP solution and CD-NP solution that was incubated in 37 C for 24 hours in HCaSMCs for 4 hours via colormetric bromodeoxyuridine(BrdU), *p <0.05

6.2.3 Effects of multiple dose CD-NP on HCaSMCs

From the earlier section (6.2.2.3), we learnt that the CD-NP's inhibitory capability is limited to 4 hours *in vitro*. However, prolonged inhibitory effect is desired. This prompted us to study if multiple doses of CD-NP can sustain or amplify the inhibition effects.

To understand if inhibitory effect is sustained with multiple dosing, we added CD-NP every 4 hours and every 8 hours. Figure 6.10 shows the relative DNA synthesis of HCaSMCs with multiple administering of CD-NP 4 hourly or 8 hourly. Although the “4h + 4h + 4h” and “4h + 8h” formulations showed decline in relative DNA synthesis compared to the control, the difference was not statistically significant.

Figure 6-10 Relative DNA synthesis of single versus multiple doses of CD-NP in HCaSMCs via colormetric bromodeoxyuridine(BrdU), *p <0.05

To further verify the actions of CD-NP on SMCs, we carried out a real time study using the xCELLigence system to understand the real time HCaSMCs proliferation. Figure 6.11 and 6.12 shows the graphs of cell index (CI) of HCaSMCs against time, where CI increment denotes increase in cell proliferation. Prior to treatment with CD-NP, cells were seeded and medium-deprived for one day each. All pre-treatments were carried out before 0 days and represented as negative days on the graph. After 24 hours of medium-deprived starvation, cells displayed unanimous decline in CI (Day -1 to day 0).

Figure 6.11 shows the cell viability of HCaSMCs with single dose CD-NP administered at concentrations of 37 $\mu\text{g/mL}$, 0.37 $\mu\text{g/mL}$ and 0.0037 $\mu\text{g/mL}$ compared to control. For the single dose CD-NP, the CI profile was similar to the control which indicates that single administration of CD-NP did not have observable effects on SMCs.

In figure 6.12, we investigated the cell viability of HCF with daily dose of CD-NP administered at concentrations of 37 $\mu\text{g/mL}$, 0.37 $\mu\text{g/mL}$ and 0.0037 $\mu\text{g/mL}$. The CI profile of multiple doses CD-NP was similar to control which indicates that multiple administration of CD-NP did not have observable effects on HCaSMCs proliferation. It appears that regardless of single or multiple doses, the CI trends are similar to the control, signifying the lack of inhibition.

Figure 6-11 Cell Index (CI) measurement of control compared to single dose infusion of different CD-NP concentrations in HCaSMCs at 0 day via the RTCA xCELLigence

Figure 6-12 Cell Index (CI) measurement of a) daily infusion of different CD-NP concentrations compared to control in HCaSMCs compared to control via the RTCA xCELLigence

6.3 Effects of released CD-NP on HCaSMCs

6.2.1 Formulation selection

From section 6.2, we learnt about the effects of pure CD-NP on HCaSMCs and these observations are summarized as follows:

- CD-NP elicit cGMP evaluation in HCaSMCs
- CD-NP's inhibition of HCaSMCs DNA synthesis inhibition is limited to 4 hours

- xCELLigence proliferation data showed lack of inhibitory effects

Upon establishing the understanding of pure CD-NP, we moved on to investigate the following:

1. Is encapsulated CD-NP bioactive?
2. Does addition of excipients affect the bioactivity of released CD-NP?
3. Does continuous release of CD-NP from films elicit different inhibitory profiles?

With the above investigation objectives, we selected the following formulations as listed in table 6.1. Please refer to chapter 4 for more details on the characterization and properties of selected formulations.

Table 6-1 Formulations to be tested for HCaSMCs studies

	Co-solvent	Excipient	Percentage	CD-NP
Films	system	addition	excipient	loading
1	Ethanol/DCM	nil	0%	1 wt%
2	Ethanol/DCM	PEG	10%	1 wt%
3	Ethanol/DCM	copolymer	10%	1 wt%

6.2.2 Elevation of cGMP production in HCaSMCs by released CD-NP

cGMP assay was carried out to test if encapsulated peptide retained the ability to produce cGMP similarly to the native CD-NP. Figure 6.13 showed the cGMP levels produced by the CD-NP released from the different polymer formulations compared to the control group. Both the pure PCL formulation group and the groups with addition of excipient showed statistically significant production of cGMP compared to the control.

Figure 6-13 Cyclic 3'5' guanosine monophosphate (cGMP) generation in HCaSMCs induced by 24 hour CD-NP release from PCL films, 10% PEG and 10% copolymer, * $p < 0.05$ versus control

6.2.3 Effects of released CD-NP on HCaSMCs proliferation

Chapter 6 *In vitro* studies on human coronary smooth muscle cells (HCaSMCs) inhibition

Next, the effect of released CD-NP on DNA synthesis of HCaSMCs was investigated. CD-NP released at 1 day, 3 days and 7 days time-points were tested. Figure 6.14 shows the relative proliferation of SMCs of the control compared to the encapsulated peptide released over a period of 7 days. Encapsulated peptide up to 7 days appears to be able to suppress DNA synthesis compared to the control in a statistically significant fashion ($P < 0.05$), this suggest that encapsulated peptide retained inhibitory capability. When comparing the inhibitory effect between different formulations, there was no statistical significance between the groups, suggesting that addition of excipients neither enhance nor decline the inhibitory effects. Furthermore, when inhibitory effects of peptide between different time-points were compared, absence of statistical significance suggests that CD-NP inhibitory effect did not deteriorate over time.

Figure 6-14 Relative DNA synthesis of CD-NP released from different CD-NP loaded formulation (released in 1 day, 3 days and 7 days) in HCaSMCs via colorimetric bromodeoxyuridine (BrdU), * $p < 0.05$

6.2.4 Effects of released CD-NP on HCaSMCs viability

Next, we investigate the effects of continuous CD-NP release from films on the viability of HCaSMCs. In figure 6.15, we investigated the real time cell viability of HCaSMCs with different film formulations. The CI profile of CD-NP released from PCL films, films with addition of 10% PEG addition and 10% copolymer addition were found to be similar to the control. This indicates that continuous administration of CD-NP via film release did not have observable inhibition on HCaSMCs proliferation.

Figure 6-15 Cell Index (CI) measurement of control compared to CD-NP released from different CD-NP loaded formulation in HCaSMCs via the RTCA xCELLigence

6.4 Discussion

CD-NP on HCaSMCs

NPs had been reported to exert their physiological effects on HCaSMCs by binding with membrane receptors, inducing cellular responses through the elevation of intracellular cGMP in a similar fashion to HCF. The *in vitro* cell culture studies carried out on HCaSMCs found that CD-NP exhibited stimulation of cGMP production more pronouncedly compared to control groups without CD-NP. This observation suggests that CD-NP functioned like its native NP in eliciting the elevation of cGMP in HCaSMCs [68, 75]. A dose-dependent relation was observed in CD-NP elicited cGMP production, which is parallel to dose-dependent elevation of cGMP by native NPs reported in literature.

In literature, the inhibitory effects of CD-NP had been reported to possess anti-proliferation properties and effective in inhibiting human aortic smooth muscle cells proliferation [125]. The study showed that CD-NP was able to inhibit smooth muscle cells for 24 hours at concentration ranges of 10^{-8} M to 10^{-5} M. However, our studies showed that the period of CD-NP inhibition on *in vitro* HCaSMCs is limited to 4 hours; despite the variation of FBS amount, experimental parameters and using ET-1 as stimulant. Furthermore, we attempted to understand the reason behind the short inhibitory effects. We found that the decline in inhibition was not due to loss of bioactivity from incubation under in 37 °C environment because we verified that CD-NP that was incubated for 24 hours in 37 °C had comparable inhibitory effects of fresh CD-NP. Hence, the author does not know the reason behind the discrepancy between the limited CD-NP inhibition observed in experiment and the reported literature.

Multiple doses CD-NP on HCaSMCs

Our results showed that single dose CD-NP inhibited in vitro HCaSMCs effectively for 4 hours. As sustained inhibition is desirable, we studied the effects of multiple dosing to understand if it would result in amplified or sustained inhibition. However, the addition of CD-NP every 4 hours for 12 hours was ineffective in prolonging the inhibitory effects. Hence, for the study of CD-NP on HCaSMCs, we concluded that inhibitory effects were limited to 4 hours. With this conclusion, we further explored the effects of released CD-NP and designed subsequent experiments based on 4 hours peptide-HCaSMCs interaction.

Released CD-NP on HCaSMCs

Very often, the manufacturing process causes proteins and peptides to undergo unintentional alteration, resulting in decline or loss of pharmacological functions and effects [207, 208]. We wanted to understand if encapsulated CD-NP retained its bioactivity. Additionally, we wanted to verify if the addition of excipients resulted in any changes to the bioactivity of encapsulated CD-NP.

The peptides released from PCL formulation was tested with the cGMP assay and we found that released peptide elicit elevation of cGMP, implying that bioactivity was retained. Next, we chose 10 % PEG addition and 10 % copolymer addition formulations to understand if the addition of excipients exerted any effects on the encapsulated peptide. CD-NP released from excipient addition formulation displayed elevation of cGMP.

Chapter 6 In vitro studies on human coronary smooth muscle cells (HCaSMCs) inhibition

Studies have reported that the addition of PEG could protect proteins and peptides from exposure of organic solvents and aid in the retention of bioactivity [207, 208]. However, it could not be verified if PEG addition had enhancing effects to bioactivity retention. But we confirmed that there is no loss of bioactivity due to the addition of PEG or copolymers. This implies that the addition of excipients could be used as a means to tailor release profiles without compromising bioactivity.

Since CD-NP inhibition in HCaSMCs was limited to 4 hours, subsequent bioactivity studies were also carried out within the 4 hours range. Peptide released at 1 day, 3 day and 7 days were collected and added to the HCaSMCs for 4 hours. We found that peptide release up to 7 days retained inhibitory effects on HCaSMCs, which implied that encapsulated CD-NP retained bioactivity up to 7 days.

Implications

Scientific implication

We observed limited inhibitory effects in HCaSMCs compared to HCF (in chapter 5). A possible explanation for this observation is that there may be more active CD-NP clearance in HCaSMCs than HCF, perhaps due to presences of more NPR-Cs.

Practical implication

From our studies, CD-NP exhibit limited inhibitory effects of 4 hours inhibition on HCaSMCs. In the practical sense, this is undesirable. However, there had been numerous reports reporting different responses to therapeutic agents in vivo HCaSMCs and in vitro HCaSMCs. Hence, to verify if CD-NP is clinically useful, verification with in vivo studies may be essential.

Chapter 7 Conclusions and Recommendations

7.1 Conclusions

In this section, we draw conclusions to the work in chapter 4, 5 and 6 in sections 7.1.1, 7.1.2 and 7.1.3 respectively. Finally, an overall conclusion would be drawn on the entire work in section 7.1.4.

7.1.1 Formulation and film studies

In chapter 4, we focused on tackling challenges in the encapsulation and delivery of CD-NP from polymeric films. Due to CD-NP's *intrinsic hydrophilicity*, we proposed the usage of co-solvent systems to overcome this issue. We investigated 2 types of co-solvent system—that is co-solvent system 1: ethanol/DCM and co-solvent system 2: water/DCM. The former co-solvent system is miscible while the latter co-solvent system is immiscible

Miscible co-solvent system 1: Ethanol/DCM

We started examining the ethanol/DCM co-solvent system first. In neat PCL films loaded with CD-NP, we achieved sustained release till 30 days. PCL films displayed a small initial release followed by subsequent release and finally releasing 97 % of the drugs by 30 days. Next, we investigated the effects of excipient addition on CD-NP release. The usage of excipients as blends is straight forward and serves two major

purposes. Firstly, the addition of hydrophilic excipient PEG resulted in a proportional increase in the initial and final release. This observation was due to physical and chemical changes associated with the addition of excipients [159, 161, 198-200]. The SEM micrograph of films with excipient addition had observable pores or dimples on the surface of films, suggesting that the formation of micro-cavities within the matrix, which provided alternative pathways for the peptides to exit. Secondly, variability of profiles could be controlled based on amount of excipients added. The peptide release is believed to be associated to the peptides leaching out along with the excipients from the correlation between the mass loss and peptide release.

Immiscible co-solvent system 2: Water/DCM

Next, we investigated the water/DCM co-solvent system. Due to immiscibility nature of DCM and water, time-dependent solution turbidity was observed. Films manufactured from short emulsification (SE) and long emulsification (LE) conditions were investigated on their peptide release, physical properties of films and distribution of peptide in the films.

The emulsification duration was found to significantly alter the initial release in PCL formulation, where peptide release from the LE films was 5-fold higher than SE films. Upon investigating the morphology, higher degree of porosity in films formed using longer emulsification duration was observed. We believe that the longer stir duration led to more porous structure overall, due to a preferential early and faster loss of the more volatile solvent from the system [206]. This porosity probably facilitated the exit of the peptide, where it desorbed rapidly upon immersion in buffer. From the CLSM, we discovered huge peptide aggregates in the LE films, whilst the lack of aggregation

in SE films suggests that peptide is homogenously dispersed throughout the film. These observations implied that the emulsification duration changed the porosity and peptide distribution, resulting in the different release kinetics between SE and LE films. Next, we investigate how the addition of excipients under different emulsification condition affects the peptide release. We observed different trends under different emulsification condition, where excipients added under SE condition increased the initial release and excipients added under the LE condition decreased in the initial release. This disparity was found to be associated to the formation of greater porosity and peptide aggregation for the SE condition, whilst decrease in porosity was observed for the LE condition when excipient was added.

We also cross-compared the release profiles of films manufactured via SE and LE. We observe that upon addition of excipients, similar release profiles was observed despite the different emulsification conditions. This observation was further verified and supported by the calculations of f_2 similarity factors. We believe that the addition of excipient acted as release kinetic stabilizer under varied emulsification. Furthermore, from the analysis of similarity factors, the copolymer addition formulation observed greater similarity between release profile manufacture from different emulsification duration. This implies that for applications involving immiscible co-solvent system, the addition of copolymers with appropriate chain lengths and hydrophilicity is a viable option to ensure consistency in therapeutic agent release profiles.

7.1.2 In vitro human cardiac fibroblast cells (HCFs) study

In chapter 5, we studied the effects of CD-NP on the inhibition of cardiac fibroblast. Since cardiac fibroblast is responsible for producing extra-cellular matrix (ECM) proteins during LV remodeling, the inhibition of fibroblast cells would prevent collagen accumulation and fibrosis.

CD-NP on HCF

In our study, we investigated the effects of single dose and multiple doses CD-NP on HCF hyperplasia and hypertrophy via the BrdU DNA synthesis assay and the real-time xCELLigence system. The BrdU assay was first used to assess the DNA synthesis in HCF. In single dose CD-NP, the inhibition of DNA synthesis was limited to 1 day, beyond which it loses suppressive capabilities. In the daily dose formulation, the suppression of DNA synthesis in HCF extended for 3 days for the lower CD-NP concentrations and extended for 5 days for the highest CD-NP concentration. This suggests that providing multiple doses of CD-NP at appropriate concentration is necessary for sustained suppression of HCF hyperplasia. Next, the xCELLigence system was used to dynamically monitor HCF spread and proliferation. In the single dose CD-NP formulation, we found that the CI trends showed no different from control group. In the multiple doses CD-NP formulation, inhibition of HCF occurred after the 3rd dose for the higher CD-NP concentrations. This implies that multiple dosing is essential for effective inhibitory of hypertrophic HCF. Additionally, we found that CD-NP elicited elevation of cGMP production which is parallel to literature reports that CD-NP exerted anti-fibrotic actions through the elevation of cGMP.

Controlled release on HCF

Upon elucidating the extent of CD-NP's inhibitory capabilities, we moved to understand the effects of CD-NP released from polymeric matrices on the HCF. We selected 3 profiles with fast, moderate and slow initial release. The three profiles were selected to understand if the release profile impacted the effectiveness in inhibition of the HCF.

Films with fast and slow initial release showed early and sustained inhibitory effects on HCF hypertrophy. The results from these two films suggest that the performance of a sustained supply of CD-NP at lower dose surpassed that of a high-dose daily bolus infusion, which we believe might be associated with the short elimination half-life of CD-NP (18.4 ± 1.4 minutes) [119]. However, the film with moderate initial release showed an absence of inhibition in the beginning but eventually express inhibition. The results seem to hint that CD-NP encapsulated in water/DCM system (films with fast and slow initial release) possessed superior bio-activity compared to the ethanol/DCM system (film with moderate release). The results suggest that the choice of co-solvent system for encapsulating CD-NP is essential for effective and inhibition effects.

7.1.3 In vitro human coronary smooth muscle cells (HCaSMCs) study

In chapter 6, we aimed to understand the effects of CD-NP and CD-NP released from films on HCaSMCs. Since HCaSMC inhibition is the main factor in mitigating

restenosis, we investigated the inhibition effects of CD-NP on HCaSMCs in this chapter.

CD-NP on HCaSMCs

In our study, CD-NP exhibited elevation of cGMP production in HCaSMCs. This observation suggests that CD-NP functioned like its native NP in eliciting the elevation of cGMP in HCaSMCs [68, 75]. Next, we investigated the effects of CD-NP on DNA synthesis in HCaSMCs. The results showed that CD-NP inhibition on in vitro HCaSMCs is limited to 4 hours. The results differed from literature report and we do not know the reason. In an attempt to understand the reason behind the limited inhibition, we studied the variation in experimental parameters including FBS amount, cell seeding density and the addition of ET-1 as stimulant. Moreover, the addition of multiple doses of CD-NP every 4 hours was investigated and found to be ineffective in prolonging the inhibitory effects. Hence, for the rest of the studies and experiments were based on 4 hours peptide-HCaSMCs interaction.

Released CD-NP on HCaSMCs

To understand if excipient addition enhanced or decline the bioactivity of encapsulated CD-NP, we investigated formulation with the addition of 10 % PEG and addition of 10 % copolymer compared to pure PCL formulation. The peptides released from all 3 formulation were tested with the cGMP assay and we found that released peptide from all formulations elicit elevation of cGMP, implying that bioactivity was retained. Further inhibition studies were carried out and CD-NP released at 1 day, 3 days and 7 days were found to retained inhibitory effects on HCaSMCs, which implied that encapsulated CD-NP retained bioactivity up to 7 days. Since we confirmed that there

was no loss of bioactivity due to addition of PEG or copolymers, the addition of excipients could be used as a means to tailor release profiles without compromising bioactivity.

7.1.4 Overall conclusion

In this section we would like to review the primary objectives of this work and draw conclusions to hypothesis and results observed.

The first objective of this work was to study and understand CD-NP release kinetics from polymer PCL and its blends. In chapter 4, we developed polymer matrices for releasing CD-NP via two different co-solvent systems. In the miscible co-solvent system, we found that addition of excipients could be used to tailor and adjust initial release of CD-NP. And in the immiscible co-solvent system, we found that the use of excipient under different emulsification condition could be used as a stabilizer. Our hypothesis of the different roles the excipients played in different systems was further supported by the morphology, mass loss and peptide distribution studies.

The second objective of our work was to understand the actions of CD-NP on cardiac cells HCaSMCs and HCFs. In chapter 5 and 6, we studied the effects of CD-NP on HCF and HCaSMCs proliferation respectively. In both HCF and HCaSMCs, we found that CD-NP induced elevation of intracellular cGMP. In chapter 5, the inhibitory effect of single dose CD-NP on hyperplasia HCF was effective for 24 hours. We studied the effects of multiple doses and found that the inhibition of hyperplasia HCF sustained up to 5 days for the highest concentration. We also investigated the effects of CD-NP on

hypertrophic HCF and found that multiple doses inhibited CD-NP after administering the 3rd dose while single dose CD-NP was ineffective in inhibiting hypertrophic HCF. In chapter 6, the inhibitory effects of CD-NP on HCaSMCs was investigated and found to be limited to 4 hours. Furthermore, multiple doses CD-NP formulation was unable to sustain the inhibitory effects. We concluded that CD-NP had limited inhibitory effects on in vitro HCaSMCs and further studies with released CD-NP was carried out within the 4 hours interaction period.

The third objective of our work was to study the effects of controlled release CD-NP on HCFs and HCaSMCs. In chapter 5 and 6, CD-NP from release formulations were found to elevate cGMP levels in both HCF and HCaSMCs. Upon verifying the retention of bioactivity of released CD-NP, we moved to understand the effects of continuous CD-NP release on HCF. In chapter 5, we discovered that continuous release of CD-NP from the films cast from water/DCM formulations yielded superior inhibition of hypertrophic HCF compared to the daily dosing of CD-NP of higher concentration. This result suggests that sustained release CD-NP from films or devices could be potentially used in treatment of moderating scar fibrotic formation and cardiac remodeling. In chapter 6, we investigated the effects of encapsulated CD-NP on HCaSMCs. We learnt that the addition of excipients did not result in loss of bioactivity in the released CD-NP. This indicates that the addition of excipients could be used as modifier or stabilizer of release profiles. However, sustained inhibition of HCaSMCs was not possible with multiple dosing or from films that released CDNP. It is possible that the dosage for sustained inhibition is well above the range studied here.

The novel contributions of this work are listed:

- 1) Development of polymer matrix for the encapsulation and sustained release of CD-NP.
- 2) Elucidated the roles of excipients in matrix formed from ethanol/DCM system encapsulating CD-NP.
- 3) Elucidated the roles of excipients in matrix formed from water/DCM system encapsulating CD-NP under two different emulsification conditions.
- 4) Understood and established the extent of CD-NP inhibition on HCF, in particular distinguished the roles of CD-NP in hyperplasia and hypertrophic HCF.
- 5) Elucidated and understood the difference between daily dose CD-NP and continuous supply of CD-NP from polymeric films on the inhibition of HCF.
- 6) Elucidated and understood the effects of CD-NP released from polymeric films on the inhibition of hyperplasia and hypertrophic HCF.
- 7) Understood and established the extent of CD-NP inhibition on HCaSMCs in systematic study of different experimental parameters.
- 8) Elucidated the effects of CD-NP released from polymeric films with and without excipient addition on the inhibition of HCaSMCs.

7.2 Recommendations

There are several directions in which the current work could be extended. The author has divided them into three broad areas: (1) Materials, (2) biological/cellular and (3) application.

7.1.1 Materials perspective

7.2.1.1 Tailoring of release profile

In this work, we used PEG (2k) and PCL (5k)-PEG (1.5k) as excipients to blend into the system. In the 2nd co-solvent system 2 (ethanol/DCM) described in chapter 4, the addition of excipients modified the peptide distribution within the film. And the addition of copolymer yield more consistent peptide distribution despite the different emulsification, however the release profiles of the peptide were still considered different. The author hypothesizes that if appropriate chain lengths of the PCL-PEG copolymer were used, it is possible to attain similar release profile despite different emulsification conditions. Moreover, it would be interesting to see how varying of PEG-block chain and PCL-block chain would impact the peptide release and the properties of the films.

7.2.1.2 Quantify peptide distribution

In chapter 4, we presented the distribution of peptide using the auto-fluorescence properties of the peptide and excipients via CLSM. We managed to see the co-localization of peptide with the excipients which supported our argument of peptide leaching out along with excipients as a mode of release kinetics. However, since the

auto-fluorescence could only pick up peptide or excipients aggregation, we were unable to see sparingly dispersed peptide or excipient. Hence, the author proposed that peptide and excipients to be fluorescently tagged. Since CD-NP has a C-terminal carboxylic acid group, this could be a point of contact for the attachment of the fluorescein. Whilst for PEG and its respective copolymer, the PEG end could be activated by using derivatives with functional groups, an example would be the usage of cyanuric chloride. For instance, CD-NP could be tagged using rhodamine-B whilst PEG and copolymer could be tagged using fluorescein isothiocyanate (FITC). This would allow us to quantify the degree of co-localization as well as the sizes of the distributions.

7.1.2 Biological and cellular perspective

7.2.2.1 CD-NP downstream signaling pathways

In chapters 5 and 6, we investigated the effects of CD-NP on HCF and HCaSMCs, particularly in the actions of CD-NP upon binding with the NP receptors and the aftermath where inhibitory effects via inhibition of cellular hyperplasia or hypertrophy. These studies were no doubt useful in giving us the indication of bioactivity and physiological effects. However, end-points of the experiments were at far time-lines (i.e cGMP experiments were carried out after 30 minutes interactions while BrdU and xCELLigence experiments were carried out in days), it was difficult to correlate the results observed. Although it has been well established from literature that the cGMP elevation resulted in inhibitory effect, it would be extremely exciting if

the signaling path from the cGMP production to the inhibitory effect could be investigated and elucidated. We know that cGMP activates protein kinase, ion channels and phosphodiesterase, hence we could isolate and investigate different factors and pathways to elucidate which is the dominant cascading effect.

7.2.2.2 Specific interactions of CD-NP with each NPR

From the experiments in chapter 5 and 6, we found that CD-NP exerted greater inhibitory actions on HCF compared to HCaSMCs. We suspected that this could be due to the presence of more NPR-Cs or circulating NEPs in HCaSMCs, resulting in greater clearance of CD-NP. Hence, studies pertaining to the measurements of NPR-Cs and NEP concentrations in HCaSMCs and HCF could be carried out. We also suspected that there is a lack of guanylyl cyclase activity in HCaSMCs compared to HCF. To verify this hypothesis, we could isolate the NPR sites and understand the interaction of CD-NP with individual NPRs. Such studies would also give us more understanding on the different pathways CD-NP exerts inhibitory actions.

7.2.2.3 Real-time imaging

From the xCELLigence experiments in chapter 5 and 6, we were able to study the real-time cell viability of HCFs and HCaSMCs. However, the xCELLigence system does not allow visual observation of the cells; hence it would be an enhancement if real-time imaging experiment could be carried out to confirm the happenings visually. Moreover, the xCELLigence system is not able to directly decouple the cell

proliferation from the cell migration. Hence, the use of a time-lapsed microscopy could help elucidate information on cell migration.

7.1.3 Development of application

7.2.3.1 Development of cardiac patches

In chapter 5, we have seen that CD-NP is a potent inhibitor of HCF proliferation and hypertrophy. Moreover, from the series of studies carried out, we learn that a continuous supply of CD-NP is necessary to ensure sustained inhibition. The potential of developing CD-NP releasing devices as a means to counteract cardiac remodeling is evident. The author propose the use of fiber spinning to construct cardiac patch meshes since such scaffold construct could facilitate more integration with the heart.

7.2.3.2 In vivo investigations

Naturally, the next step would be to put these cardiac patches to test. For the in vivo evaluation, the author proposed to start from small animal models, where rat model with left coronary ligation could be investigated. The CD-NP releasing patch could be compared to the commercial dacron patches and PCL polymer patches without CD-NP. In this study, the LV cavity area, wall thickness, growth factor expression and histology should be studied. The research outcome would give an indication of the performance from the clinical point of view and provide preliminary knowledge to further formulation developments.

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