Biomimetic coacervates for drug delivery

Lim, Zhi Wei

2020

Lim, Z. W. (2020). Biomimetic coacervates for drug delivery. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/137527>

<https://doi.org/10.32657/10356/137527>

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0).

Downloaded on 26 Apr 2023 23:23:09 SGT

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

BIOMIMETIC COACERVATES FOR DRUG DELIVERY

LIM ZHI WEI

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2020

BIOMIMETIC COACERVATES FOR DRUG DELIVERY

LIM ZHI WEI

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2020

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

06-Nov-2019

.....
Date

A handwritten signature in black ink, appearing to read 'Lim Zhi Wei', positioned above a dotted line.

.....
LIM ZHI WEI

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

06-Nov-2019

.....
Date

.....
Prof Ali Miserez

Authorship Attribution Statement

Parts of Chapters 4 and 5 are published as Zhi Wei Lim, Yuan Ping, and Ali Miserez, Glucose-Responsive Peptide Coacervates with High Encapsulation Efficiency for Controlled Release of Insulin, *Bioconjugate Chemistry* **2018** 29 (7), 2176-2180

DOI: 10.1021/acs.bioconjchem.8b00369

The contributions of the co-authors are as follows:

- Prof Ali Miserez and Dr Ping Yuan provided the initial project direction and revised the manuscript drafts.
- I prepared the manuscript draft.
- I performed all the laboratory work at the Research Techno Plaza in NTU. All the data were also analysed by me.
- All microscopy, including sample preparation, was performed by me in the Imaging facility located at Singapore Centre for Environmental Life Sciences Engineering.

Parts of Chapter 4 and 6 are published as Zhi Wei Lim, Vijaykumar B. Varma, Raju V. Ramanujan and Ali Miserez, Magnetically Responsive Peptide Coacervates with Dual Triggered Hyperthermia and Chemotherapy for Liver Cancer Treatment. Submitted, *Biomaterials Science*.

The contributions of the co-authors are as follows:

- Prof Ali Miserez and Prof Raju provided initial project directions and edited the manuscripts draft.
- The drafts of the manuscript are written by me. The manuscript was revised together with Dr Vijaykumar.
- I performed all the experiments except magnetic studies of coacervates under AMF.
- Magnetic studies of coacervates were performed by Dr Vijaykumar at MSE.
- Confocal microscopy of cells was performed by me in the Imaging facility located at Singapore Centre for Environmental Life Sciences Engineering.

06-Nov-2019

.....
Date

A handwritten signature in black ink, appearing to read 'Lim Zhi Wei', with a stylized, cursive script.

.....
LIM ZHI WEI

Abstract

Biomimetics has provided inspirations to design innovative materials and advanced technologies. The squid beak of *Dosidicus gigas*, commonly known as Humboldt squid is a fascinating instance, being a “biotool” with remarkable gradient stiffness, yet fully organic and non-mineralised. The four main components of the squid beak are chitin, pigments, water, and proteins. There are two families of proteins within the squid beak, chitin binding beak proteins (DgCBPs) and histidine-rich beak proteins (DgHBPs). DgHBPs have been demonstrated to undergo coacervation. Among the tested recombinant DgHBPs and biomimetic peptides, DgHBP-2 peptide was found to be most suitable as an encapsulation material, due to its ability to coacervate under physiological conditions, its pH and temperature responsive coacervation. With the aim of exploring DgHBP-2 peptide coacervates as drug delivery systems, characterisation of the coacervates was first done to understand its properties and limitations. Different crosslinking methods were also developed with the aim of stabilising these coacervates.

The next study attempts to incorporate DgHBP-2 peptide coacervates into Glucose Responsive Insulin Delivery System (GRIDS). In this GRIDS, Glucose oxidase (GOx) and insulin were both loaded into DgHBP-2 peptide coacervates. GOx functions as a glucose sensor while the coacervates acted as an insulin reservoir. GOx hydrolyses glucose into gluconic acid, bringing about a localised acidic microenvironment that causes the disassembly of coacervates and release of insulin. The encapsulation efficiency of insulin by DgHBP-2 peptide coacervates was more 99% and was found to be cytocompatible. *In vitro* release assays have shown that the amount and rate of insulin release were influenced by the surrounding glucose concentration, with higher glucose concentration triggering faster release of insulin. This coacervate-based GRIDS could also respond to varying glucose concentrations that mimics blood glucose fluctuations within human body. In addition, the structure of insulin released from coacervates was near identical to the native insulin, indicating that its bioactivity was not affected. Together, these results have shown that coacervate-based GRIDS can be a potential insulin replacement therapy for diabetes.

With the successful GRIDS fabrication, the use of coacervates for thermo-chemotherapy for liver cancer was explored next. The effectiveness of chemotherapies in liver cancer are often limited by its severe toxicity and hence chemotherapeutic drugs e.g. Doxorubicin (Dox) should be targeted towards the tumour site. Furthermore, hyperthermia treatments have been shown to improve the efficacy of chemotherapy, decreasing the need to use high concentrations of chemotherapeutic drugs. Magnetic nanoparticles and Dox were co-encapsulated within DgHBP-2 coacervates, followed by crosslinking with 4-methylcatechol and sodium periodate. These Dox-loaded Magnetic coacervates (DMCs) were efficiently uptaken into HepG2 liver cancer cells through an ATP- and endocytosis-independent pathway. Application of an external Alternating current Magnetic Field (AMF) on DMCs increased the temperature to 45°C within the coacervates, destabilizing the droplets and triggering the release of Dox. *In vitro* studies on DMCs-treated HepG2 cells have shown that they were cytocompatible and capable of triggering cell death under AMF. Moreover, thermo-chemotherapy treatments by DMCs were found to be more effective than either hyperthermia or chemotherapy treatments alone.

This thesis highlights the use of biomimetic peptide coacervates as a novel natural encapsulation material. By performing *in vitro* studies on DgHBP-2 peptide coacervates, their integration into drug delivery system was examined more comprehensively, from the encapsulation of therapeutics to release assays and cytotoxic studies. The experiments presented in this thesis provide proof-of-concept validation that stimuli-responsive coacervate microdroplets can be used environmental-responsive drug delivery systems.

Lay Summary

The study of biological materials has provided many useful inspirations to create bio-inspired, innovative materials. The squid beak of *Dosidicus gigas* (Humboldt squid) is an interesting biotool that exhibits unique biomechanical features such as a mechanical property gradient from the proximal region inserted in the buccal mass of the animal to the distal hard tip used to chew on prey. Unlike vertebrates' bones and teeth that rely on calcium phosphate (apatite) mineralisation for its strength, the beak is made only of organic biopolymers, namely chitin (the second most common polysaccharide in Nature after cellulose) and proteins. The most abundant family of beak proteins, Histidine rich beak proteins (DgHBPs), have been shown to undergo a phenomenon called coacervation under specific physico-chemical conditions, during which they separate into two liquid phases: one dilute phase with low concentration of proteins, and one concentrated phase in the form of liquid-like microdroplets, also called "coacervates". These liquid microdroplets exhibit the ability to package other types of biomolecules (of both low- and high-molecular weight) and can thus be used to package drugs. Destabilizing the coacervates after drug encapsulation thus allows for the development of smart drug delivery systems, as explored in this thesis. Smart drug delivery systems are means of transporting drugs to specific parts of the body. It can also be defined as drug carriers capable of controlling the rate of drug release through an external stimuli. Among the different proteins and peptides tested in this study, DgHBP-2 peptide was found to be the most suitable as a drug delivery carriers due to its pH and temperature responsive properties.

Diabetic patients suffer from side effects due to insulin incorrect dosage in response to glucose content in the bloodstream. One way to tackle this issue is to design insulin delivery systems that can respond to glucose ("Glucose Responsive Insulin Delivery System", GRIDS) and release insulin only at high glucose concentration but not at normal glucose concentration. In this thesis, a GRIDS was designed that consisted in packaging the glucose-sensing enzyme GOx as well as insulin into DgHBP-2 peptide liquid microdroplets. GOx converts glucose to gluconic acid, which acidifies the microenvironment within the liquid microdroplets, leading to dissolution of the

microdroplets and in turn to insulin release. The loading capacity for insulin in these liquid droplets was extremely high (>99%) and was also found to be non-toxic. The amount and rate of insulin release were dependent on the glucose concentrations. More importantly, this system could respond to glucose concentration fluctuations, commonly seen in human blood glucose levels. These results have shown that liquid microdroplets-based GRIDS can be potentially used to control blood glucose in diabetic patients.

The use of chemotherapy to treat liver cancer often results in serious side effects, due to the use of toxic anti-cancer drug e.g. Doxorubicin (Dox). The toxicity of these drugs arises because of non-specific interactions with healthy cells. To increase the efficacy and reduce the toxicity of chemotherapy, anti-cancer drugs should only be delivered to tumour site. In addition, thermal therapy, also known as hyperthermia has been shown to improve the effectiveness of chemotherapy, even directly killing cancer cells in certain cases. To develop a delivery system capable of both chemotherapy and hyperthermia, Dox and magnetic nanoparticles (MNPs) were packaged into DgHBP-2 peptide microdroplets. Dox acted as an anti-cancer drug while MNPs generated heat (~45°C) for hyperthermia upon exposure to Alternating current Magnetic Field (AMF). Application of AMF destabilized the micro-droplets. Thus triggering the release of Dox, which combined with hyperthermia induced significant HepG2 liver cancer cell death. This combined therapy was found to be more effective in inducing cell death when compared to either hyperthermia or chemotherapy.

From the above two proof-of-concept studies, peptide-based coacervate microdroplets have been established to represent suitable biocompatible packaging material for stimuli-responsive drug carriers. A better understanding on the encapsulation properties of these microdroplets has been gained, demonstrating that a large palette of cargo molecules can be packaged, from low molecular compounds to protein therapeutics to MNPs. Drug release profiles triggered by external stimuli was also established, as well as cell culture studies demonstrating the cytocompatibility of these peptide coacervates. Moreover, these studies pave the way for further development and optimization in the design and fabrication of other coacervate microdroplets-based delivery systems.

Acknowledgements

First, I want to express my greatest gratitude and appreciation to my supervisor, Professor Ali Miserez from the School of Materials Science and Engineering (MSE), Nanyang Technological University (NTU) for his support, valuable advices and guidance during this project. I very grateful for the opportunity provided by Professor Ali Miserez in joining his research group, involving me in his collaboration projects, allowing me to gain more research experience. The questions and suggestions asked during our lab meeting and group discussions have deeply influenced me to be a better scientist.

Secondly, I want to extend my appreciation to Dr Ping Yuan for his generous guidance in the project. With his extensive experience in the field of drug delivery research, Dr Ping Yuan has encouraged and inspired me in many ways during my Ph.D. journey. His valuable advice and creative ideas have also helped me in different phases of the project.

Thirdly, I also wish to thank Professor Lam Yeng Ming and Professor Raju V. Ramanujan from MSE,NTU for being on my Thesis Advisory Committee, give me vital mentorship and supervision in the preparation of my thesis.

I would like to thank Professor Raju V. Ramanujan, Professor Czarny Bertrand and Dr Varma Vijaykumar Babulalji for their great guidance in our collaboration projects.

Heartfelt appreciation is extended to all who has helped me in this project: Dr Hiew Shu Hui, Mr Loke Jun Jie, and Ms Clarinda Sutanto for their wonderful help with experiments and equipment training.

Special thanks to Dr Bartosz Gabryelczyk and Dr Cai Hao for the advice given on coacervation studies and characterisation.

I want to thank the MSE and NTU for providing scholarship to support this project.

Finally, I am grateful to my family members and wife, Hui Ling for supporting me during my pursuit of Ph.D. studies.

Table of Contents

Abstract	i
Lay Summary	iii
Acknowledgements	v
Table of Contents	vii
Table Captions	xv
Figure Captions	xxxiii
Abbreviations	xxxv
 Chapter 1 Introduction.....	 1
1.1 Hypothesis /Problem Statement	4
1.1.1 Problem Statement	4
1.1.1 Hypothesis.....	6
1.2 Objectives and Scope	6
1.3 Dissertation Overview.....	6
1.4 Findings and Outcomes/Originality	8
References.....	9
 Chapter 2 Literature Review	 13
2.1 Overview	14
2.1.1 Sucker Ring Teeth.....	15

2.1.2	<i>D.gigas</i> squid beak and its composition	16
2.1.3	Mechanical properties of <i>D.gigas</i> beak	18
2.1.4	Inspired materials from <i>D.gigas</i> beak	20
2.1.5	Extraction and identification of <i>D.gigas</i> beak proteins (DgBPs).....	23
2.1.6	Sequence features and primary structure analysis of DgHBPs.....	26
2.1.7	Secondary structure characterisation of DgHBP-1 and 2	28
2.1.8	Coacervation conditions of DgHBPs	31
2.1.9	Time-dependent structural changes of DgHBPs coacervates	34
2.1.10	Coacervation of DgHBP-derived peptides.....	35
2.2	Overview of diabetes and glucose responsive insulin delivery system (GRIDS)..	36
2.2.1	Glucose sensing by enzymatic method	37
2.2.2	Diol-binding chemicals	41
2.3	Overview of cancer and targeted drug delivery	44
2.3.1	Biomedical applications of Magnetic Nanoparticles (MNPs)	45
2.3.2	Targeted delivery using MNPs	46
2.3.3	Targeted delivery by co-encapsulation	46
2.3.4	Targeted delivery by Drug-conjugated MNPs.....	48
2.3.5	Magnetic hyperthermia (MHT).....	49
2.4	Questions to answer based on literature	53
2.4.1	Glucose responsive delivery system	53
2.4.2	MNPs and Thermo-chemotherapy	54
2.5	PhD in context of literature	55
	References	56

Chapter 3	Experimental Methodology	65
3.1	Characterisation and crosslinking of coacervates	66
3.1.1	Relative turbidity for coacervation studies	66
3.1.2	Optical and fluorescence microscopy	67
3.1.3	Dynamic light scattering	67
3.1.4	Solid phase peptide synthesis (SPPS) of DgHBP-2 peptide DOPA	68
3.1.5	Reverse-Phase High performance liquid chromatography (RP-HPLC)	70
3.1.6	Matrix assisted laser desorption or ionisation- time of flight mass spectrometry (MALDI-TOF)	71
3.1.7	Arnow staining	72
3.1.8	Crosslinking of DgHBP-2 peptide DOPA coacervates	73
3.1.9	Crosslinking of DgHBP-2 peptide coacervates	74
3.1.10	Field Emission Scanning Electron Microscopy (FESEM)	75
3.1.11	Transmission Electron Microscopy (TEM)	76
3.1.12	Zeta potential	77
3.1.13	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay	77
3.1.14	Live/dead staining	78
3.2	Glucose responsive insulin delivery system	79
3.2.1	Labelling of Insulin and GOx	79
3.2.2	Encapsulation of drug by coacervation	80
3.2.3	Fluorescence emission assay for insulin-FITC and Dox	80
3.2.4	Encapsulation efficiency (EE%) (Fluorescence method)	80
3.2.5	Encapsulation efficiency (EE%) (RP-HPLC method)	81
3.2.6	<i>In vitro</i> insulin release assay	81
3.2.7	Circular Dichroism (CD) Spectroscopy	82

3.2.8	Enzyme-linked Immunosorbent Assay (ELISA)	83
3.3	Dox-loaded magnetic coacervates (DMCs)	84
3.3.1	Magnetic alignment of coacervates	84
3.3.2	Confocal microscopy	85
3.3.3	Endosomal staining with lysotracker).....	86
3.3.4	Heating mechanism of MNPs under AMF	87
	References	88

Chapter 4 Characterisation and crosslinking of coacervates93

4.1	Introduction	94
4.2	Experimental Methods	95
4.2.1	Relative turbidity for coacervation studies	95
4.2.2	Optical microscopy	95
4.2.3	DLS measurements	95
4.2.4	Solid phase peptide synthesis (SPPS)	95
4.2.5	RP-HPLC	96
4.2.6	Arnow staining.....	97
4.2.7	Maldi-TOF	97
4.2.8	Crosslinking of DgHBP-2 peptide DOPA coacervates	97
4.2.9	Crosslinking of DgHBP-2 peptide coacervates	98
4.2.10	Scanning Electron Microscopy (SEM)	98
4.2.11	Transmission Electron Microscopy (TEM)	98
4.2.12	Zeta potential	98
4.2.13	MTT assay	98
4.2.14	Live/dead staining	99

4.3	Characterisation and crosslinking of DgHBP-2 peptide coacervates	99
4.3.1	Description of tested biomimetic peptides and proteins	99
4.3.2	Relative turbidity of biomimetic peptides and recombinant proteins	100
4.3.3	Microscope images of coacervates	103
4.3.4	Size of DgHBP-2 peptide coacervates	104
4.3.5	Reversible coacervation of DgHBP-2 peptide	106
4.3.6	Synthesis of DgHBP-2 peptide DOPA	108
4.3.7	Purification and mass analysis of DgHBP-2 peptide DOPA	109
4.3.8	Characterisation of DgHBP-2 peptide DOPA	110
4.3.9	Crosslinking of DgHBP-2 peptide DOPA coacervates	111
4.3.10	DOPA-Fe crosslinked coacervates	112
4.3.11	DOPA-NaIO ₄ crosslinked coacervates	114
4.3.12	Crosslinking of DgHBP-2 peptide coacervates	116
4.3.13	Dityrosine crosslinked coacervates	117
4.3.14	4-Methyl catechol (4-MC)/NaIO ₄ crosslinked coacervates	118
4.3.15	Zeta potential of DgHBP-2 coacervates	120
4.3.16	<i>In vitro</i> cytotoxic assay on NIH3T3 fibroblasts	121
4.4	Conclusions	123
	References	124
Chapter 5	Glucose responsive insulin delivery system	127
5.1	Introduction	128
5.2	Experimental methods	129
5.2.1	Labelling of Insulin and GOx	129
5.2.2	Maldi-TOF	130

5.2.3 Drug Loading into coacervates	130
5.2.4 Fluorescence and optical microscopy	130
5.2.5 Fluorescence emission assay for insulin-FITC	130
5.2.6 Encapsulation efficiency (Fluorescence method)	130
5.2.7 Encapsulation efficiency (RP-HPLC method).....	131
5.2.8 MTT assay	131
5.2.9 In vitro release assay (Glucose responsive time point assay).....	131
5.2.10 In vitro release assay (alternating glucose concentration assay)	131
5.2.11 Circular Dichroism (CD) Spectroscopy	132
5.2.12 Enzyme-linked Immunosorbent Assay (ELISA)	132
5.3 Insulin and GOx-loaded coacervates	133
5.3.1 Labelling and encapsulation of insulin-FITC	133
5.3.2 Encapsulation efficiency of insulin-FITC by DgHBP-2-peptide coacervates	134
5.3.3 GOx for sensing glucose	136
5.3.4 <i>In vitro</i> cytotoxic assay on NIH3T3 fibroblasts	137
5.3.5 <i>In vitro</i> release of insulin triggered by glucose	137
5.3.6 Activity of insulin after encapsulation and release	140
5.4 Conclusions	141
References	142
 Chapter 6 Magnetically Responsive Peptide Coacervates with Dual Triggered Hyperthermia and Chemotherapy for Liver Cancer Treatment	145
6.1 Introduction	146
6.2 Experimental Methods	149
6.2.1 Materials	149

6.2.2	Encapsulation of Dox and MNPs by coacervates	149
6.2.3	Fluorescence and optical microscopy	149
6.2.4	Magnetic alignment of coacervates	149
6.2.5	Encapsulation efficiency	150
6.2.6	Concentration of Dox within coacervates.....	150
6.2.7	Crosslinking of DgHBP-2 peptide coacervates	150
6.2.8	Dynamic light scattering (DLS).....	151
6.2.9	MTT assay	151
6.2.10	Live/dead staining	151
6.2.11	Scanning Electron microscopy	151
6.2.12	Transmission Electron Microscopy	151
6.2.13	Fluorescence recovery after photobleaching (FRAP)	151
6.2.14	Lysotracker staining.....	151
6.2.15	Uptake assay with endocytosis inhibitors	152
6.2.16	<i>In vitro</i> release assay (Temperature triggered Dox release)	152
6.2.17	Temperature measurement of coacervates under AMF	153
6.2.18	Cumulative release of Dox over time	153
6.2.19	Cumulative release of Dox by multiple AMF cycles	153
6.2.20	Specific absorption rate (SAR)	153
6.2.21	Intrinsic loss of power (ILP)	153
6.3	Doxorubicin-loaded magnetic coacervates	154
6.3.1	Encapsulation of Dox by DgHBP-2 peptide coacervates	154
6.3.2	Crosslinking of DMCs by different methods.....	156
6.3.3	Cellular uptake of coacervates	164
6.3.4	<i>In vitro</i> heat triggered release of Dox	167

6.3.5	Cytotoxicity efficacy of released Dox from DMCs.....	170
6.3.6	Magnetic studies of crosslinked DMCs for thermo-chemotherapy	172
6.4	Conclusion.....	179
	References.....	180
Chapter 7	Discussion and Future Work	185
7.1	General discussion.....	186
7.1.1	Characterisation of DgHBP-2 peptide coacervates.....	186
7.1.2	Glucose responsive insulin delivery system	187
7.1.3	Magnetically Responsive Peptide Coacervates with Dual Triggered Hyperthermia and Chemotherapy for Liver Cancer Treatment.....	190
7.2	Reconnaissance work for future studies.....	191
7.2.1	Encapsulation of therapeutic peptides	191
7.2.2	Encapsulation of CRISPR-Cas9 system	193
7.2.3	Uptake mechanism of DgHBP-2 peptide coacervates	194
7.2.4	<i>In vivo</i> biodistribution studies of coacervates.....	197
7.3	Outlook and Future work	199
7.3.1	Relationship between peptide sequences, length and coacervation properties	200
7.3.2	<i>In vivo</i> biodistribution studies of coacervates.....	200
7.3.3	Diabetic animal models studies	200
7.3.4	Liver cancer animal model.....	201
7.3.5	Cellular uptake mechanism of coacervates.....	202
7.3.6	Peptide delivery and Transfection studies	203
	References.....	204

Table Captions

- Table 2.1** Sequences of DgCBPs and techniques used to identify them.
- Table 2.2** Protein sequence of DgHBP-1, 2 and 3, with the repeat domain sequences underlined.
- Table 2.3** Molecular weight (Mw), amino acid number (A. A.), pI, and acidic/basic/aromatic residues count ($R^{\text{acidic}}/R^{\text{basic}}/R^{\text{arom}}$) for DgHBPs.
- Table 2.4** Tandem repeats within the C-terminal of DgHBPs. Underlined sequences represent conserved sequences within the repeats.
- Table 2.5.** Percentage of different secondary structures in DgHBPs obtained by FTIR analysis.
- Table 4.1** Summary of conditions that form coacervation.
- Table 5.1** EE% of insulin-FITC by 1 mg/mL of DgHBP-2-peptide coacervates. ($n = 3$, mean values $\pm$ S.D)
- Table 5.2** EE% of 0.1 mg/mL of insulin-FITC by DgHBP-2-peptide coacervates. ($n = 3$, mean $\pm$ S.D)
- Table 6.1** EE% of different concentrations of Dox by DgHBP-2 peptide coacervates. ($n = 3$, mean $\pm$ S.D.)
- Table 6.2** Concentration of Dox within DgHBP-2 peptide coacervates. ($n = 3$, mean $\pm$ S.D.)

Figure Captions

Figure 1.1 Image of SRT within the suckers of *D. Gigas*'s tentacles.

Figure 1.2 Image of *D. Gigas* and its squid beak.

Figure 2.1 Illustration of *D. gigas* and its biotools (sucker ring teeth and squid beak).

Figure 2.2 Features of *D. gigas* SRT. (A) Image of extracted SRT. (B) Higher magnification of SRT. (C) Modular architecture of suckerin-19, demonstrating alternating Ala-His and Gly-Try rich domains.

Figure 2.3 Squid beak of *D. gigas* within the soft buccal tissue.

Figure 2.4 Composition analysis of *D. gigas* beak. (A) Protein composition within different regions of squid beak. (B) Concentration of the 4 main components (chitin, protein, pigments and water) within squid beak by chemical and gravimetric analysis.

Figure 2.5 Pigmentation of *D. gigas* squid beak. (A) lateral view of upper beak without buccal tissue. (B) Pigment gradient from rostrum to wing of squid beak. (C) Dissected wing region. (D) sections of beak demonstration different intensity of pigmentation gradient.

Figure 2.6 Mechanical characterisation of *D. gigas* beak. (A) Ashby plot (Young's modulus (E) vs Hardness (H)) of squid beak with common materials. (B) Critical yielding load (H^3/E^2) vs fracture energy (G_c) of different materials. Horizontal and vertical lines were critical loads for yielding and cracking respectively. (C) Young's modulus and tensile strength in different areas of squid beak. (D) Tensile stress vs tensile strain curves for different regions of hydrated beak.

Figure 2.7 Illustration of squid beak-inspired, catechol-functionalised chitosan+ γ -Fe₂O₃ composite film.

Figure 2.8 Characterisation of chitosan+ γ -Fe₂O₃ composite film. (A) Young's modules of hydrated and dehydrated film. (B) Magnetisation curve for chitosan+ γ -Fe₂O₃ composite film. (C) Experimental setup for magnetisation studies of chitosan+ γ -Fe₂O₃ film.

Figure 2.9 Squid beak-inspired allyl-CNCs+PVAs composite film. (A) Illustration of the film casting and photo-crosslinking procedures. (B) Demonstration of hydration-enhanced mechanical gradient model in squid beak and squid-inspired composite film.

Figure 2.10 Mechanical properties of composite film. (A) Tensile storage modulus of un-crosslinked and crosslinked composite film in either dry or wet conditions. (B) Illustration of Allyl-CNC+PVAc composite film. Solid lines represent regions with increasing amount of UV exposure whereas red lines represent regions of films that were cut out for mechanical testing. Experiments were performed on samples that were wet at 37 °C, dry at 25 °C and dry at 80 °C (C) Twisting of composite film with gradient crosslinked film (top panel) and uniformly crosslinked gradient (bottom panel).

Figure 2.11 SDS PAGE of extracted beak proteins with different staining. Coomassie Blue R-250 (CBR) staining for all proteins; Pauly staining for His-rich proteins; NBT staining for proteins containing catechols and Lectin binding assay to identified *N*-Acetylglucosamine oligomers. (L= protein ladder)

Figure 2.12 Illustration of DgCBP and DgHBP protein family. X represents a hydrophobic residue, whereas X' is normally Tyr and X'' is usually either Gly or Ala.

Figure 2.13. CD spectrum of (A) DgHBP-1 and (B) DgHBP-2 at low (1 mg/mL) and high concentration (10 mg/mL).

Figure 2.14 CD spectrum of (A) DgHBP-1 and (B) DgHBP-2 at different temperatures.

Figure 2.15 Relative turbidity of (A) DgHBP-1 and (B) DgHBP-2 coacervates in different buffers (ionic strength and pH). NaCl was used to adjust the ionic strength of the buffers.

Figure 2.16 Optical micrographs of DgHBP-1 (left column) and DgHBP-2 (right column) coacervates over time.

Figure 2.17 DLS measurement of DgHBP-1 (top panel) and DgHBP-2 (bottom panel) coacervates over time.

Figure 2.18 Rheology results of DgHBPs and its consensus peptides. (A) Viscosity of DgHBPs vs shear rate. (B) Storage (G') and viscous(G'') modulus of DgHBPs. (C) Viscosity of DgHBPs consensus peptides (pepHBP-1 and pepHBP-2) vs shear rate. (D) Storage (G') and viscous(G'') modulus of pepHBPs vs shear rate.

Figure 2.19 CD spectrum of (A) DgHBP-1 and (B) DgHBP-2 coacervates over time.

Figure 2.20 Coacervation of DgHBP peptide in phosphate buffer. (A) Relative turbidity at different pH and ionic strength. (B) Light micrograph of DgHBP peptide coacervates. ($n = 3$, technical replicates, error bars represent mean $\pm$ s.d.)

Figure 2.21 Illustration of a closed-loop insulin delivery method. This delivery system is capable of sensing the glucose level while releasing the correct amount of insulin at the same time. Once the glucose level goes back to normal, insulin release will stop.

Figure 2.22 Chemical reaction of glucose to gluconic acid catalysed by GOx. GOx oxidises glucose into D-glucono- δ -lactone, which further hydrolyses to gluconic acid.

Figure 2.23 Glucose-responsive microgel loaded with GOx and Insulin. (A) Illustration of a glucose-responsive microgel loaded with both GOx and Insulin. GOx converts glucose into gluconic acid resulting in a localised decrease pH. The subsequent

protonation results in microgel swelling and release of insulin. **(B)** Insulin is only released in the presence of high glucose concentration (400 mg/dL) but not in low glucose concentration (100 mg/dL) or PBS.

Figure 2.24 Illustration of glucose-responsive gating membrane on a porous PVDF membrane substrate. GOx converts glucose to gluconic acid and decreases the local pH. The protonation of the carboxyl groups results in the shrinking of PAAC chains and thus to the opening of PVDF membrane pores.

Figure 2.25 Illustration of glucose-responsive nanovesicle loaded with insulin, GOx and catalase. **(A)** Conversion of glucose to gluconic acid acidifies inner core of nanovesicle, resulting in dissociation and release of its contents. **(B)** Chemical structure of Ser-Ketal and its hydrolysis into PEG-polyserine at low pH.

Figure 2.26 Formation of boronate esters from PBA and diols at different pH.

Figure 2.27 Anionic boronate ester formed between PBA and glucose in aqueous solution.

Figure 2.28 Nanoparticles with glucose molecules crosslinked with PBA. Glucose competitively binds to PBA on the polymer, resulting in dissociation of the nanoparticle assemblies and release of insulin.

Figure 2.29 Chemically modified insulin with addition of a glucose-like diol and PBA molecule. **(A)** Chemical structure of the modified insulin. **(B)** Illustration of aggregation between the chemically modified insulins and its dissociation in the presence of glucose.

Figure 2.30 Biomedical applications of MNPs. **(A)** Analytical applications. **(B)** Therapeutic applications.

Figure 2.31 Reconstituted high-density lipoprotein (rHDL) nanoparticles for

magnetic targeted delivery. (A) Illustration of rHDL-valrubicin-SPION nanoparticles. (B) Prostate cancer cells were treated with rHDL-valrubicin-SPION nanoparticles, followed by Prussian blue staining to reveal MNPs. Left: Cells are subjected to directional magnetic field, before staining; Middle: cells that are near to magnetic field; Right: cells that are farther from magnetic field.

Figure 2.32 Characterisation of MNP+Dox loaded PLGA nanoparticles. (A) TEM micrograph. (B) *In vitro* Dox release profiles at pH 5 and pH 7.4. ($n = 3$, average) (C) Percentages of live, apoptotic and necrotic cells when treated with MNPs only, free Dox only and Dox+MNPs PLGA nanoparticles. (D) Mean LLC tumour volume after different treatments. ($n = 6$ to 10, mean $\pm$ S.D.)

Figure 2.33 Dual coating of SPION for greater drug loading capacity. (A) SPION was first coated with SiO₂, then a subsequent second layer of mPEG-PLA polymer was coated onto Fe₃O₄-SiO₂ to form the final Fe₃O₄-SiO₂-Polym. (B) Dual coated SPION had higher drug loading compared to single coated SPION. The loading capacity increases with the thickness of second coating. ($n = 3$)

Figure 2.37 Combination therapy using moderate hyperthermia and TRAIL-loaded hydrogel. (A) Illustration of gelation and dissolution of hydrogels at different temperature. Nanocomplex solution undergo gelation upon reaching 37 °C, trapping TRAIL and SPION within the hydrogel structure. Magnetic hyperthermia (MHT) increased the temperature within the hydrogel, inducing dissolution and release of TRAIL. (B) Tumour volume after different T/S-NHs and hyperthermia treatments. ($n = 4$) (C) TUNEL staining of tumour tissues treated with T/S-NHs +/- MHT. Green fluorescence indicates cells that were undergoing apoptosis.

Figure 2.38 Characterisation of Dox-loaded MNPs-PMs. Temperature measurements of MNPs-PM under AMF, with water acting as control. (B) Rate of Dox release from MNPs-PMs without AMF, with AMF and at 45 °C. (C) Cell viability of A549 cells treated with MNPs-PMs, free Dox or Dox-loaded MNPs-PMs in the presence or absence of AMF.

($n=3$, biological repeats, mean $\pm$ S.D.) A p -value of < 0.05 was considered to be statistically significant.

Figure 3.1 General peptide synthesis by SPPS method. C-terminus of the last amino acid is coupled to the solid support resin through a linker. The peptide is then elongated by cycles of i) Deprotection of N-terminus group (NPG); ii) activation of carboxy group, and iii) coupling of next amino acid to the previous one. The final polypeptide and SPG are then cleaved off from the resin. Aa and SPG represent amino acid and side-chain protecting group.

Figure 3.2 Ionisation of sample molecules in Maldi-TOF.

Figure 3.3 Coordination and covalent crosslinking of catechols.

Figure 3.4 Mechanisms of dityrosine crosslinking by riboflavin and UVA. (UVA: ultraviolet light A; ISC: intersystem crosslinking and RF: riboflavin)

Figure 3.5 Chemical reaction of isothiocyanate with biomolecules to form thiourea.

Figure 3.6 Characteristic CD spectra of different secondary structures.

Figure 3.7 Principle of sandwich ELISA assay. Monoclonal antibodies specific for the antigen of interest are coated onto the wells. After the sample is added to the wells, antigens within the sample will bound to the antibody. Enzyme-linked secondary antibodies are then added to the wells. These secondary antibodies will bind to the antigens and remained within the well after washing. Substrate is next added and converts to coloured product by antibody-bounded enzyme. (E: enzyme and S: substrate)

Figure 3.8 Different classification of endocytosis in mammalian cells. CME and CIE represent clathrin-mediated endocytosis and clathrin-independent endocytosis respectively.

Figure 3.9 Mechanisms of heat generation (MHT) by MNPs in AMF.

Figure 4.1 Sequence of biomimetic peptides: pepHBP-1, DgHBP-2-peptide and Mfp3s-peptide.

Figure 4.2 Optical image of plastic cuvettes containing phosphate buffer (left) and DgHBP-2 peptide coacervates (right)

Figure 4.3 Relative turbidity of peptide and protein coacervates (1 mg/mL). (A) DgHBP-1 (B) DgHBP-2 (C) DgHBP-2 peptide (D) pepHBP-1. The turbidity assays were done at 25°C and 5 mins after the onset of coacervation. ($n = 3$, mean $\pm$ S.D.)

Figure 4.4 Coacervation studies of Mfp3s-peptide. (A) Relative turbidity of Mfp3s-peptide coacervates. (B) Micrograph of Mfp3s-peptide coacervates. White arrows represent coacervates that were attached onto the glass coverslip. The turbidity assays and imaging were done at 25°C and 5 mins after the onset of coacervation. ($n = 3$, mean $\pm$ S.D.)

Figure 4.5 Micrographs of peptide and protein coacervates (1mg/ml). (A) pepHBP-1 (pH 8.5, 2.0 M ionic strength); (B) DgHBP-2 peptide (pH 7.5, 0.1 M ionic strength); (C) DgHBP-1 protein (pH 6.5, 0.1 M ionic strength); (D) DgHBP-2 protein (pH 7.5, 0.1 M ionic strength). White arrows represent coacervates that were attached onto the glass coverslip. Microscopy of the coacervates was done at 25°C and 5 mins after the onset of coacervation.

Figure 4.6 DLS measurements of DgHBP-2 peptide coacervates over different timepoint. (A) Diameter of DgHBP-2 peptide before coacervation in 10 mM acetic acid. (B) Effective diameter of DgHBP-2 peptide coacervates from 0 min to 40 min. ($n = 5$, mean $\pm$ S.D.)

Figure 4.7. Merging of fluorescein-loaded and rhodamine B-loaded DgHBP-2 peptide coacervates. (A) Micrograph of the two differentially-loaded coacervates immediately after mixing (0min). White arrows represent coacervates loaded with either RITC or FITC. (B) Size distribution analysis of the coacervates at 0 min by ImageJ software. (C) Micrograph of the two differentially-loaded coacervates after mixing and incubated at room temperature for 60 min. White arrowhead represents coacervates undergoing merging.

(D) Size distribution analysis of the coacervates after incubation for 60 min by ImageJ software.

Figure 4.8. Time-lapse video showing reversible coacervation of DgHBP-2 peptide. DgHBP-2 coacervates were first allowed to attach onto the glass coverslip ($T = 0s$). Upon addition of 0.1M HCl ($T = 3s$), the coacervates started to dissociate due to the decrease in pH ($T = 4.5s$). The coacervates were fully dissociated at $T = 9s$. Upon addition of 0.1M NaOH to increase the pH ($T = 13s$), the coacervation process occurred again once the buffer pH was between 7.5 to 9.5 ($T = 43s$, white arrow). At $T = 55s$, micrograph of DgHBP-2 showed that the coacervates attached onto the surface of glass coverslips.

Figure 4.9 Stability of DgHBP-2 peptide coacervates (1 mg/mL) at different temperature. ($n = 3$, mean $\pm$ S.D.)

Figure 4.10 SPPS of DgHBP-2 peptide DOPA. RP-HPLC of DgHBP-2 peptide DOPA by C18 column (A) before purification (brown dotted box: peak with desired peptide) (B) after purification. (C) Theoretical molecular weight of DgHBP-2 peptide DOPA with different number of DOPA amino acids. (D) Molecular weight of DgHBP-2 peptide DOPA by MALDI-TOF.

Figure 4.11 Characterisation of DgHBP-2 peptide DOPA. (A) DgHBP-2 peptide DOPA and DgHBP-2 peptide solution after Arnow staining. Colour change is observed only in DgHBP-2 peptide DOPA. (B) Absorbance spectrum of DgHBP-2 peptide DOPA after Arnow staining. Absorbance was increased only in samples that contained catechol groups (DgHBP-2 peptide DOPA and DOPA amino acids). (C) Relative turbidity of DgHBP-2 peptide DOPA coacervates at 1 mg/mL. ($n = 3$, mean $\pm$ S.D.) (D) Micrograph of DgHBP-2 peptide DOPA coacervates at 1 mg/mL.

Figure 4.12 Illustration of DgHBP-2 peptide DOPA coacervation and subsequent crosslinking.

Figure 4.13. DgHBP-2 peptide DOPA-Fe crosslinked coacervates. (A) Micrograph (left) and optical image of plastic cuvette (right) containing DOPA-Fe crosslinked coacervates. (B) DLS of DOPA-Fe crosslinked coacervates. (C) Upon additional of 25 mM EDTA to the DOPA-Fe crosslinked coacervates, the coacervates collapsed immediately and only aggregates were observed in the micrograph. (D-E) DOPA-Fe crosslinked coacervates were next subjected to phosphate buffers of different pH. At $\text{pH} \leq 4.5$ (D) and $\text{pH} \geq 10$ (E), the crosslinked coacervates dissociated, indicating that they are only stable at pH between 5 to 9.5. The EDTA and pH experiments were done at 25°C.

Figure 4.14. Electron microscopy of DOPA-Fe crosslinked coacervates. (A, B) SEM micrographs. (C, D) TEM micrographs. (B and D were electron micrographs of DOPA-Fe crosslinked coacervates taken at a higher magnification in SEM and TEM respectively. White and red arrows represent coacervates of different sizes. The web-like net structure (green arrows) seen under the coacervates in the TEM micrographs is the lacey carbon support film that is part of the TEM copper grids.

Figure 4.15. DgHBP-2 peptide DOPA-NaIO₄ crosslinked coacervates. (A) Micrograph (left) and optical image of plastic cuvette (right) containing DOPA-NaIO₄ crosslinked coacervates. (B) DLS of DOPA-NaIO₄ crosslinked coacervates ($n = 5$) (C) Upon additional of 25 mM EDTA to the DOPA-NaIO₄ crosslinked coacervates, the crosslinked coacervates remained stable as observed in the micrograph. (D-E) DOPA- NaIO₄ crosslinked coacervates were next subjected to buffers of different pH (1 to 13). The crosslinked coacervates remained stable at all tested buffers. The EDTA and pH experiments were done at 25°C.

Figure 4.16. Electron microscopy of DOPA-NaIO₄ crosslinked coacervates. (A, B) SEM micrographs. (C, D) TEM micrographs. (B and D were electron micrographs of DOPA-NaIO₄ crosslinked coacervates taken at a higher magnification in SEM and TEM respectively. White and red arrows represent coacervates of different sizes, whereas blue arrows indicate the white spots seen on the surface of crosslinked coacervates. The web-

like net structure (green arrows) seen under the coacervates in the TEM micrographs is the lacey carbon support film that is part of the TEM copper grids.

Figure 4.17 Illustration of DgHBP-2 peptide coacervation and subsequent crosslinking.

Figure 4.18 Dityrosine crosslinking of DgHBP-2 peptide using riboflavin as a photoinitiator. (A) Fluorescence analysis of dityrosine crosslinked peptide. (B) Micrograph of dityrosine crosslinked peptide coacervates in deionised water after centrifugation.

Figure 4.19 DgHBP-2 peptide coacervates crosslinked with 4-MC and NaIO₄. (A) Micrograph (left) and optical image of plastic cuvette (right) containing 4-MC/NaIO₄ crosslinked coacervates. (B) DLS of 4-MC/NaIO₄ crosslinked coacervates. ($n = 5$)

Figure 4.20. Electron microscopy of 4-MC/NaIO₄ crosslinked coacervates. (A, B) SEM micrographs. (C, D) TEM micrographs. (B and D were electron micrographs of 4-MC/NaIO₄ crosslinked coacervates taken at a higher magnification in SEM and TEM respectively. White and red arrows represent coacervates of different sizes, whereas purple arrow indicates the budding effect from the crosslinked coacervates. The webbed-like net structures (green arrows) seen under the coacervates in the TEM micrographs are the lacey carbon support film that are part of the TEM copper grids.

Figure 4.21 Zeta potential measurement of DgHBP-2 peptide coacervates. (A) phosphate buffer (pH 7.5, 0.1M) (B) PBS (C) DgHBP-2 peptide coacervates (D) DOPA-Fe crosslinked coacervates (E) DOPA-NaIO₄ crosslinked coacervates (F) 4-MC/NaIO₄ crosslinked coacervates. ($n = 5$)

Figure 4.22. Relative cell viability of NIH3T3 mouse fibroblast treated with (A) DgHBP-2 peptide, (B) DOPA-Fe crosslinked coacervates, (C) DOPA-NaIO₄ crosslinked

coacervates, and **(D)** 4-MC/NaIO₄ crosslinked coacervates. Control cells (without treatment) were normalised to 100%. ($n = 6$, mean $\pm$ S.D.)

Figure 4.23 Live/dead staining of NIH3T3 fibroblast cells treated with coacervates. **(A)** Control live cells, **(B)** control dead cells (killed by 70% methanol), **(C)** DgHBP-2 peptide coacervates, **(D)** DOPA-Fe crosslinked coacervates, **(E)** DOPA-NaIO₄ crosslinked coacervates, and **(F)** 4-MC/NaIO₄ crosslinked coacervates.

Figure 5.1 Illustration of GRIDS created from DgHBP-2 peptide coacervates. **(A)** Coacervation and encapsulation of insulin and GOx. **(B)** Once the insulin +GOx loaded coacervates are subjected to glucose, the glucose enters the coacervates and hydrolyses to gluconic acid. This leads to a localised pH decrease within the coacervates, which subsequently cause the coacervates to dissociate and releases the insulin.

Figure 5.2 Purification of insulin-FITC and its encapsulation by DgHBP-2 peptide coacervates. **(A)** Maldi-TOF results of human insulin labelled with FITC. **(B)** Micrographs of DgHBP-2 peptide coacervates (1 mg/mL) loaded with insulin-FITC (0.1 mg/mL). Light micrograph (left); fluorescence micrograph (middle); merged micrograph (right). The green fluorescence and grey droplets were insulin-FITC and DgHBP-2 peptide coacervates respectively.

Figure 5.3 RP-HPLC absorbance chromatograms of **(A)** DgHBP-2 peptide in 10 mM acetic acid (1 mg/mL); **(B)** native insulin (0.1 mg/mL); **(C)** supernatant after removing the insulin-loaded coacervates; and **(D)** dissolved coacervates pellet after removing supernatant. Red arrow specifies the retention time of native insulin. After insulin encapsulation, virtually no insulin was detected in the supernatant, whereas after dissolving the coacervates pellet, insulin was detected within the coacervates. These data further confirmed the high EE% of insulin by coacervates as measured by fluorescence spectroscopy (Table 5.1 and 5.2).

Figure 5.4 Micrographs of DgHBP-2 peptide coacervates (1 mg/mL) loaded with GOx-RITC (0.1 mg/mL) and insulin-FITC (0.1 mg/mL). (A) Green fluorescence micrograph represents insulin-FITC. (B) Red fluorescence micrograph represents GOx-RITC. (C) Light microscopy micrograph represents DgHBP-2 peptide coacervates. (D) Merged (green, red, and light) micrograph. Yellow fluorescence within coacervates confirmed co-encapsulation of GOx-RITC and insulin-FITC.

Figure 5.5 Relative cell viability of coacervates treated NIH3T3 fibroblasts. (A) DgHBP-2 peptide coacervates, and (B) Insulin-loaded DgHBP-2 peptide coacervates. Control cells (without treatment) were normalized to 100%. ($n = 6$, mean $\pm$ S.D.)

Figure 5.6 Glucose triggered release of insulin from DgHBP-2 peptide coacervates in phosphate buffer (A) Insulin loaded coacervates with 4 mg/L of glucose (circle, red); insulin loaded coacervates without glucose (inverted triangle, pink); GOx+insulin coacervates without glucose (triangle, blue); and GOx+insulin coacervates with 4 mg/mL of glucose (square, black). (B) Alternate hyperglycaemic (4 mg/mL, blue) and normoglycaemic (1 mg/mL, green) solution. ($n = 3$, mean $\pm$ S.D.)

Figure 5.7 *In vitro* release assay of insulin from DgHBP-2 peptide coacervates in different glucose concentration (1 mg to 4 mg). 4 mg/mL of glucose (square, black); 3 mg/mL of glucose (inverted triangle, pink); 2 mg/mL of glucose (triangle, blue); and 1 mg/mL of glucose (circle, red).

Figure 5.8 Comparison of native and released insulin from DgHBP-2 peptide coacervates. (A) CD spectra of DgHBP-2 peptide (black line), released insulin (red line), heat denatured insulin (blue dotted line), and native insulin (Green line). (B) ELISA of released and native insulin. ($n = 3$, mean $\pm$ S.D.)

Figure 5.9 CD spectra of native insulin in mildly acidic buffers (pH 5.5 to 7.5). ($n = 3$)

Figure 6.1 Illustration of DMCs for thermo-chemotherapy. (A) DMCs are formed by encapsulation of Dox and MNPs into DgHBP-2 peptide coacervates. Dox and MNPs are first pipetted into phosphate buffer at their respective concentrations, DgHBP-2 peptide solution is next added to induce coacervation and encapsulation. (B) When DMCs reach the tumour site, MHT produced by MNPs under AMF triggers heat-induced disassembly of DMCs and ultimately release of Dox.

Figure 6.2 Magnetic alignment of DMCs under uniform magnetic field. (A) illustration of the experimental setup. (B) Magnetic strength and field distribution of the two permanent magnets on the $\pm Y$ and $+Z$ directions.

Figure 6.3 Encapsulation of Dox by DgHBP-2 peptide coacervates in different pH buffers. Left: light micrographs; Middle: fluorescence micrographs; Right: merged channels micrographs.

Figure 6.4 Micrographs of DMCs. (A) without magnetic field and (B) with magnetic field.

Figure 6.5 Fluorescence micrograph of crosslinked DMCs by different methods. (A) un-crosslinked DMCs, (B) DOPA-FeCl₃ crosslinked DMCs, DOPA-NaIO₄ crosslinked DMCs (C) without magnetic field and (D) with magnetic field.

Figure 6.6 Fluorescence micrographs of 4-MC/NaIO₄ crosslinked DMCs. (A) without magnetic field and (B) with magnetic field.

Figure 6.7 SEM micrographs of 4-MC/NaIO₄ crosslinked DMCs. (A) low magnification field and (B) higher magnification

Figure 6.8. TEM micrographs of 4-MC/NaIO₄ crosslinked DMCs. (A) low magnification field and (B) higher magnification. Red arrows and brown arrows represent DMCs and MNPs respectively; purple arrows indicate the budding effect seen on top of

DMCs. The webbed-like net structures (green arrows) seen under the coacervates in the micrographs are the lacey carbon support film that are part of the TEM copper grids.

Figure 6.9. Quantification of fluorescence signal from crosslinked DMCs in FRAP experiments ($n = 6$, $mean \pm S.D.$). Fluorescence recovery of ~80% was reached in DMCs 60s after photobleaching.

Figure 6.10. DLS of DMCs (A) before and (B-F) after crosslinking in PBS. Two distribution size of DMCs were observed before and after crosslinking, which were similar and remained stable for at least 15 days. ($n = 5$)

Figure 6.11. Fluorescence intensity of Doxorubicin (calculated concentration of Dox within DMCs) and crosslinked DMCs. Minimal loss of Dox was observed from the DMCs after crosslinking. ($n = 3$, $mean \pm S.D.$)

Figure 6.12. Fluorescence micrographs of DMCs (A) before crosslinking and (B-F) after crosslinking in PBS. Crosslinked DMCs are stable in PBS for at least 15 days without significant loss of the Dox cargo (red fluorescence).

Figure 6.13. Confocal micrographs (3D view) of HepG2 cells treated with GFP-loaded coacervates and lysotracker probes. (A, B) untreated cells. (C, D) un-crosslinked coacervates. (E, F) 4-MC/NaIO₄ crosslinked coacervates. Green fluorescence (GFP-loaded coacervates) was observed beneath the cell membrane (white box), indicating uptake of coacervates into the cells. Red and white fluorescence represents endosomes and cell membranes.

Figure 6.14. Confocal micrographs of HepG2 cells treated with GFP-loaded coacervates (100 $\mu\text{g/mL}$) and lysotracker probes. (A, D) Control cells. (B, E) GFP loaded un-crosslinked coacervates. (C, F) GFP loaded 4-MC/NaIO₄ crosslinked coacervates. Top rows: z-stack merged images. Bottom rows: z-stack merged images with membrane staining. GFP-loaded coacervates (green fluorescence, green arrow) and

endosomes (red fluorescence, red arrow) were not co-localized. White fluorescence represents the cell membrane.

Figure 6.15. Confocal micrographs of HepG2 cells treated with endocytosis inhibitors (orthographic view). (A, B) CME inhibitors chlorpromazine (30 μ M) (A) and chloroquine (100 μ M) (B). (C, D) Pinocytosis inhibitors amiloride hydrochloride (20 μ M) (C) and cytochalasin B (8.5 μ M) (D). (E) Methyl- β -cyclodextrin (1 mM), and (F) NaN_3 (10 mM). Green fluorescence and white colour represent GFP-loaded coacervates and the cell membrane, respectively.

Figure 6.16. Cellular uptake of GFP loaded coacervates by HepG2 cells at 4°C (orthographic view). (A) un-crosslinked coacervates. (B) 4-MC/ NaIO_4 crosslinked coacervates. Green fluorescence and white colour represent GFP-loaded coacervates and the cell membrane, respectively. Cells were first incubated at 4°C for 30 mins before treated with GFP-loaded coacervates. Confocal imaging was done after 2 hours of incubation with coacervates at 4°C.

Figure 6.17 Release of Dox from 4-MC/ NaIO_4 crosslinked DMCs (100 μ g/mL) at different temperatures. ($n = 3$, mean $\pm$ S.D.)

Figure 6.18. Live/dead staining of 4-MC/ NaIO_4 crosslinked DMCs treated HepG2 cells. (A) Control cells (without treatment). (B) Control dead cells (without treatment, fixed with 70% methanol). (C) Crosslinked coacervates (without Dox and MNPs, 100 μ g/mL). (D-H) crosslinked DMCs (100 μ g/mL) without pre-heating (D); 30 min of pre-heating (F); 1 hour of pre-heating (G); 2 hours of pre-heating (G); and 6 hours of pre-heating (H).

Figure 6.19. Relative cell viability of pre-heated 4-MC/ NaIO_4 crosslinked DMCs treated HepG2 cells. (A) no pre-heating. (B) 30 min of pre-heating. (C) 2 hours of pre-heating. (D) 6 hours of pre-heating. ($n = 6$, mean $\pm$ S.D.)

Figure 6.20. The heating curve of Ferrofluid and DMCs (under AMF) used in SAR calculation.

Figure 6.21. Magnetic studies of 4-MC/NaIO₄ crosslinked DMCs under AMF (A) Temperature of crosslinked DMCs over 40 mins of AMF. MNPs diluted in PBS act as positive control. (B) Cumulative release of Dox vs time during a single cycle of AMF, and at 25 °C and 37 °C in the absence of AMF. (C) Cumulative release of Dox with multiple AMF cycles (Each cycle: 20 min of AMF with 10 mins of cooling), equivalent to a local temperature of ca. 45°C. (D) Pulsatile release of Dox from DMCs by several cycles of AMF, heating the DMCs to 45°C, then cooling to room temperature (28°C). Pink and purple columns represent AMF “ON” and “OFF” respectively. Concentration of DMCs and MNPs were 500 µg/mL and 0.04% v/v, correspondingly. (*n* = 3, mean ± S.D.)

Figure 6.22. DLS and micrographs of crosslinked DMCs after AMF treatment. (A and B) 1× AMF cycle; (C and D) 2× AMF cycle; (E and F) 3× AMF cycle; (G and H) 4× AMF cycle and (I and J) 5× AMF cycle. Each AMF cycle was 20 min to achieve 45 °C, followed by cooling to 28 °C.

Figure 6.23. Live/dead staining assay on treated HepG2 live cancer cells. (A) with one AMF cycle. (B) 4-MC/NaIO₄ crosslinked DMCs without AMF cycle. (C) MNPs with 1 AMF cycle (D-F) 4-MC/NaIO₄ crosslinked DMCs with 1 AMF cycle (D); 2 AMF cycles (E); and 3 AMF cycles (F). DMCs and MNPs concentrations were 500 µg/mL and 0.04% v/v respectively. (G) Percentage of HepG2 cell death with different AMF cycles. (*n* = 3, mean ± S.D.) For MHT + Dox treatment after 3 cycles, 100% cell death was achieved.

Figure 6.24. Live/dead staining assay on MHT or Dox treated HepG2 live cancer cells. (A-C) MNPs-loaded, 4-MC/NaIO₄ crosslinked coacervates with 1 AMF cycle (A); 2 AMF cycles (B); and 3 AMF cycles (C). (D-F) Dox only treatment (Dox was first released from the 4-MC/NaIO₄ crosslinked DMCs in the cell culture medium before adding to cells) after 1 AMF cycle (D); 2 AMF cycles (E); and 3 AMF cycles (F). Concentration of DMC was 500 µg/mL.

Figure 7.1 RP-HPLC chromatogram (220nm) of (A) PM-2 peptide (0.14 mg/mL); (B) supernatant after removing PM-2 loaded coacervates; (C) VIP-71 peptide (0.37 mg/mL) and (D) supernatant after removing VIP-71 loaded coacervates.

Figure 7.2 Micrographs of human iPSCs after treated with RNA-RFP loaded coacervates. (A) 3 hours control (without coacervates); (B) Coacervates treated cells (3 hours); (C) 24 hours control (without coacervates) and (D) Coacervates treated cells (24 hours).

Figure 7.3 Interaction between GUV (doped with 0.1 mol% Rhodamine-PE) and GFP-loaded 4-MC/NaIO₄ crosslinked coacervates in 100 mM glucose solution. Left: 3D projection of deconvoluted z-stack. Right: 2D cross-sectional views of different channels.

Figure 7.4 Micrographs of FITC-DgHBP-2 peptide with GUVs. (A) Merged channel; (B) Red fluorescence and (C) Green fluorescence.

Figure 7.5 Confocal micrographs (orthographic view) of HepG2 cells treated with (A) 1 μ M and (B) 10 μ M of FITC-DgHBP-2 peptide for 24 hours.

Figure 7.6 Micrographs of cy5.5-loaded coacervates. Fluorescence micrograph (left); Optical micrograph (middle) and Merged channel micrograph (right).

Figure 7.7 Total radiant of BL6 mouse organs injected with 100 μ L of cy5.5 -loaded coacervates at 1- and 24-hour post-injection. Un-injected wildtype BL6 mice act as control.

Figure 7.8 confocal micrographs of BSA-FITC coated, cy5.5-loaded coacervates. (A and B) 3D projection of z-stack, (C) Z stack image and (D) orthographic view. Green fluorescence and red fluorescence represent BSA-FITC and cy5.5-loaded coacervates respectively.

Abbreviations

4-MC	4-methylcatechol
Ala	Alaine
Allyl-CNCs	Alkene-modified Cellulose Nanocrystals
AMF	Alternating current Magnetic Field
Asp/D	Aspartic acid
ARF	ADP Ribosylation Factor
Boc	Tert-butyloxycarbonyl
BSA	Bovine Serum Albumin
CBD	Chirin Binding Domain
CBR	Coomassie Blue R250
CCD	Charged-Coupled Device
CD	Circular Dichroism
CRISPR	Clustered Regularly Interspace Short Palindromic Repeats
<i>D. gigas</i>	<i>Dosidicus gigas</i>
Da	Dalton
DCM	Dichloromethane
DIC	N,N-Diisopropylcarbodiimide
DEN	Diethylnitrosamine
DgBP	<i>D. gigas</i> Beak Protein
DgCBP	<i>D. gigas</i> Chitin-binding Beak Protein
DgHBP	<i>D. gigas</i> Histidine-rich Beak Protein
DLS	Dynamic Light Scattering
DMAP	4-Dimethylaminopyridine
DMCs	Doxorubicin-loaded Magnetic Coacervates
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EE%	Encapsulation Efficiency
ELISA	Enzyme-Linked Immunosorbent Assay
ELP	Elastin-Like Polypeptides

Dox	Doxorubicin
DOPA	l-3,4-dihydroxyphenylalanine
FeCl ₃	Iron(III) chloride
Fe ₂ O	Iron oxide
FESEM	Field Emission Scanning Electron Microscopy
FITC	Fluorescein Isothiocyanate
Fomc	9-Fluorenylmethyloxycarbonyl
Gln/Q	Glutamine
Glu/E	Glutamic Acid/Glutamate
Gly/G	Glycine
GOx	Glucose Oxidase
GRIDS	Glucose Responsive Insulin Delivery System
GUVs	Giant Unilamellar Vesicles
H ₂ O ₂	Hydrogen Peroxide
HepG2	Human Hepatocyte Carcinoma cell line
His/H	Histidine
HPLC	High Performance Liquid Chromatography
iPSCs	Induced Pluripotent Stem Cells
kDa Kilo	Dalton
Leu/L	Leucine
MALDI-ToF	Matrix Assisted Laser Desorption Ionization-Time of Flight
MDM2	Mouse Double Minute 2 homolog
Mf3s	Mussel foot protein 3s
MNPs	Magnetic Nanoparticles
MS/MS	Tandem Mass Spectrometry
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide
NaN ₃	Sodium Azide
NaOH	Sodium Hydroxide
NaIO ₄	Sodium Periodate
NBT	Nitroblue Tetrazolium
Nd-Fe-B	Neodymium

NMR	Nuclear Magnetic Resonance
NOD	Non-Obese Diabetic
PAGE	Polyacrylamide Gel Electrophoresis
PBA	PhenylBoronic Acid
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
Phe/F	Phenylalanine
PLGA	Poly(lactic-co-glycolic acid)
Pro/P	Proline
RACE	Rapid Amplification of Complementary DNA ends
RES	Reticuloendothelial System
RITC	Rhodamine B isothiocyanate
RNA	Ribonucleic Acid
RP	Reverse Phase
PVAc	Poly-vinyl acetate
SDS	Sodium Dodecyl Sulfate
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscope
Ser/S	Serine
SPPS	Solid Phase Peptide Synthesis
SRT	Sucker Ring Teeth
TEM	Transmission Electron Microscopy
Thr/T	Threonine
TNBS	2,4,6-Triitrobenzene Sulfonic Acid
TRAIL	Tumour necrosis factor-Related Apoptosis-Inducing Ligand
Tyr/Y	Tyrosine
UV	Ultra-violet
Val/V	Valine

Chapter 1

Introduction

*Biological materials have evolved over millions of years to adapt towards their environment and perform specific functions needed for their survival. Materials biomimicry is the science of understanding biological materials with the purpose of replicating their unique strategies to solve everyday problems. The squid beak from *Dosidicus gigas* is a fascinating biological system that has attracted the attention of the biomimetic community in recent years. Problems related to encapsulation and drug delivery are briefly presented in this Chapter. Finally, this Chapter presents the hypothesis, objectives, and the summary of the main findings of the thesis.*

1.0 Introduction

Biomimicry or biomimetics is to understand natural processes, and to later replicate some of these biological principles to solve human problems.¹ Biological systems have been developed and improved through billion years of evolution and natural selection.^{1, 2} Each of these biological innovations has therefore been efficiently tuned for maximum survival in their respective environments. Hence, Nature often offers inspirations for engineering strategies that may be appropriate for the benefit of mankind. Examples of technologies inspired by design found in Nature include Velcro inspired by the hooks on ends of burrs or Shinkansen bullet train inspired from the shape of a kingfisher's beak.³⁻⁵

One marine model organism that has gained interest is *D. Gigas*, from the family of Ommastrephidae squids.⁶⁻⁸ They are also commonly known as Humboldt squid. *D. Gigas* is equipped with a sharp beak and powerful suckers, which make it one of the most efficient and ferocious predators in the Ocean. They are found mainly in the eastern Pacific Ocean and can grow up to 2 meter in length, swimming with a top speed of ~24 km/hour.⁸ The biomimetic community has been intrigued by *D. Gigas* because of its unique biotools, particularly their sucker and squid beak.⁸ Sucker Ring Teeth (SRT) are non-mineralised, protein-based teeth that are embedded within the suckers (Figure 1.1) that act as a grappling tool during predation. These SRT display outstanding mechanical properties and can be re-mold into different shapes by thermal treatment without changing their mechanical properties.^{9, 10}

Figure 1.1. Image of SRT within the suckers of *D. Gigas*' tentacles.¹¹

The squid beak is a fully organic composite (*i.e.* devoid of any calcified mineral) that has a spatial gradient of stiffness and hardness (Figure 1.2).^{7, 8} The soft proximal beak region attaches to the muscular buccal mass and, the beak becomes increasingly stiff as it extends towards the distal rostrum. This gradient of stiffness and hardness has been proposed to be regulated by the two major protein families found in the squid beak, DgCBPs and DgHBPs.^{6, 12} DgCBPs is thought to form a structural scaffold with chitin during beak maturation. DgHBPs has then been suggested to diffuse through this chitin/DgCBPs scaffold during beak growth and maturation, and to strengthen the structure by a sclerotization mechanism involving covalent inter-protein covalent crosslinking.⁶ While the exact mechanism of beak maturation is still currently unknown, it is believed that DgHBPs undergo liquid-liquid phase separation (LLPS) or coacervation to facilitate its infiltration through the DgCBPs-Chitin scaffold.⁶ Indeed, DgHBPs have been shown to undergo simple coacervation.^{6, 12} Since one of the most promising application of coacervates is encapsulation of various molecules such as omega-3 and fish oil,^{13, 14} in this project we thought to exploit DgHBP-based coacervates as potential drug delivery systems.

Figure 1.2. Image of *D. Gigas* and its squid beak.¹⁵

1.1 Problem statement/hypothesis

1.1.1 Problem statement

Drugs are used extensively to treat diseases. However, for a drug to be therapeutically effective, it must be delivered and achieved the required therapeutic dose at its target organ/tissue.¹⁶⁻¹⁹ The drug must also be clear out of its target after a certain period of time to minimise its side effects. These undesirable side effects arise because of the unwanted interaction between the drug and healthy tissue, limiting the ability to design optimal treatments for various diseases such as cancer.²⁰ While the drug is inside the blood circulation, it is susceptible to degradation and drug stability may eventually be affected.^{17, 18} Hydrophobic drugs have poor solubility and may precipitate in aqueous environment, making these drugs difficult to administrate. Therefore, controlled and targeted drug delivery systems are actively being research on to overcome these problems.²¹⁻²⁷ The goal of these 'smart' drug delivery systems is to deliver drugs to specific targets of body through a vehicle that can control drug release by physiological or chemical trigger.¹⁶⁻²⁰ The type of delivery system used will be depend on numerous factors such as type of drug, target tissue, disease being treated and route of administration. To realize this goal, researchers around the world are turning to advances in microencapsulation or nanoencapsulation to develop these drug delivery systems.^{21, 23-25, 28-30}

Micro-encapsulation in drug delivery is a process where therapeutics are enclosed within a capsule, known as microspheres.^{31, 32} The therapeutics are either encapsulated within a shell or physically trapped within a network. Polymers were first to be used as encapsulation material due to their easily tuneable chemical and physical properties, and their ease of processing.³³ Despite these advantages, production of synthetic polymers is non-environmentally friendly and residual organic solvents may have detrimental effects on the bioactivity of therapeutics.^{34, 35} Also, non-biodegradable polymers do not break down easily and need to be removed after use. On the other hand, a special class of polymers known as biodegradable synthetic polymers are synthetic polymers that can break down into non-toxic monomers after use.³⁶ However, the toxicity of these degradation products is always in doubt as biodegradable polymer degradation often result in a range

of different fragment sizes, making toxicity studies difficult to determine experimentally.^{33, 36} Therefore, natural polymers e.g. chitin, elastin and collagen which are usually biodegradable and biocompatible, are of great interest recently as material for encapsulation.³⁷⁻³⁹

Natural biodegradable polymers have been used in different types of encapsulation methods.^{36, 38-44} Of the different encapsulation methods, coacervation is becomingly increasingly popular, albeit less explored than other traditional methods.⁴⁵⁻⁴⁹ The advantages of coacervation over other encapsulation methods include fast self-assembly in aqueous environment, no specialised equipment needed and high loading capacity.^{50, 51} Notably, gelatin/gum Arabic and tropoelastin are used extensively in complex and simple coacervation respectively. A major limitation of complex coacervation is that it is dependent on electrostatic interactions to facilitate encapsulation and may not be suitable for encapsulation of many proteins as their charge is dependent on buffer condition and sequence. Drug delivery systems based on tropoelastin coacervates are not suitable to be used for heat triggered drug release such as thermo-chemotherapy. This is due to tropoelastin-coacervation are based on hydrophobic interactions, where an increased in temperature increases the level of tropoelastin coacervation^{52, 53} and ultimately decrease the amount of drug release. Hence, new materials that are capable of coacervation are required.

Recently, two families of beak proteins from *D. gigas* have been identified and sequenced, namely chitin-binding beak proteins and histidine-rich beak proteins.^{6, 12} Chitin-binding proteins function as a physical support to guide and position chitin polymers, whereas histidine-rich beak proteins undergo simple coacervation for efficient delivery into their specific location through the chitin network during beak maturation. Three histidine-rich beak proteins are identified (DgHBP-1, DgHBP-2, and DgHBP-3) from the squid beak. The C-terminal of these proteins comprise of tandem His- and Gly-rich repeats that are important for coacervation process.^{6, 12}

1.12 Hypothesis

The overall hypothesis of this thesis is that recombinant proteins or biomimetic peptides coacervates derived from beak protein of *D. gigas*, can be a potential biocompatible material for encapsulation of therapeutics. DgHBPs coacervates are *D. gigas*'s solution to encapsulate and transport highly concentrated beak proteins through the chitin network during beak maturation, hence making DgHBPs an attractive material for fabrication of drug delivery system. Other than beak proteins, coacervates based from DgHBP may be able to carry other molecules such as peptides, small molecule drugs and higher molecular weight proteins. These drug-loaded coacervates can then be subsequently used in controlled release and targeted drug delivery systems.

1.2 Objectives and scope

This thesis aims to experimentally demonstrate the use of coacervates derived from *D. gigas* as encapsulation material and incorporate these coacervates into delivery systems.

- Characterisation of protein and peptide coacervates, followed by determining the optimal protein or peptide as encapsulation material.
- Design and fabricate a glucose/pH responsive insulin delivery system for controlled release of insulin.
- To generate and manipulate magnetically responsive coacervates for targeted and controlled release of Dox.
- Study the encapsulation efficiencies and release profiles of the above delivery systems. *In vitro* cell culture assays will be done to assess the toxicity and uptake of these delivery systems.

1.3 Dissertation and Overview

There are seven chapters in this thesis and they are described briefly below:

Chapter 1: Introduction

This chapter states the problems in materials for encapsulation and drug delivery. The overall hypothesis and objectives of this project are next presented in this chapter. This chapter also includes the dissertation overview and innovative findings.

Chapter 2: literature review

This chapter reviews the literature on squid beak of *D. gigas*, DgHBPs coacervates and smart delivery systems for diabetes and cancer treatments.

Chapter 3: Experimental methodology

This chapter describes the methods and principles of the experiments used in this study.

Chapter 4: Characterisation and crosslinking of coacervates

This chapter studies the optimum buffer conditions for coacervation of DgHBPs and biomimetic peptides. DgHBP-2 peptide is found to be optimal for encapsulation material. Different crosslinking methods followed by characterisation and toxicity studies of these crosslinked coacervates are also included.

Chapter 5: Glucose responsive insulin delivery system

This chapter establishes the use of coacervates to fabricate a glucose responsive delivery system for controlled release of insulin. Results pertaining to characterisation of this delivery system such as encapsulation efficiency, cytotoxicity, release profiles and secondary structure of released insulin are also included in this chapter.

Chapter 6: DMCs for thermo-chemotherapy

This chapter describes the use of magnetic coacervates for controlled release of Dox under AC magnetic field. Besides the characterisation assays, cell uptake of these coacervates are included in this chapter as well.

Chapter 7: Discussion and Future work

This chapter summaries and discuss the main results obtained in this project. Suggestions for future work are also presented.

1.4 Findings and Outcomes/Originality.

This study has led to several significant and novel outcomes:

1. Most of previous studies on simple coacervation based delivery system were done using tropoelastin or elastin like polypeptides. In this study, biomimetic peptide coacervates derived from beak protein of *D. gigas* have been successfully used to prepare simple coacervates for 2 different drug delivery systems.
2. While many different forms of drug delivery systems such as hydrogel and nanoparticles has been researched extensively for controlled delivery of insulin, this is for the first time that simple coacervates have been used for the fabrication of GRIDS. With a simple yet fast co-encapsulation of insulin and GOx, coacervates-based GRIDS can respond to changing glucose levels *in vitro* and release insulin accordingly. The structure of released insulin is also found to be similar to native insulin, indicating the retention of its bioactivity.
3. DgHBP-2 peptide coacervates are easily uptake by HepG2 cells and this uptake process is endocytosis- and ATP-independent. Cellular uptake is only observed when DgHBP-2 peptide is assembled into coacervates and not as peptide molecules, suggesting that coacervation is crucial to the uptake process. Since the coacervates are not inside the endosomes after cell uptake, any drug within the coacervates can be triggered to release into the cytoplasm directly, avoiding lysosomal degradation.
4. Majority of the polymers used in thermo-responsive drug delivery systems are synthetic polymers such as poly(N-isopropylacrylamide). Natural thermo-responsive polymers such as elastin-like polypeptides and methylcellulose are few and hyperthermia drives the assembly of these natural polymers. In contrast, hyperthermia drives the dissociation of DgHBP-2 peptide coacervates, allowing the concurrent release of its drug contents and thermal therapy. Indeed, the effectiveness of combined therapy on HepG2 cells was higher when compared to Dox-only or hyperthermia-only treated cells.

References

1. J. Hwang, Y. Jeong, J. M. Park, K. H. Lee, J. W. Hong and J. Choi, *Int J Nanomedicine*, 2015, 10, 5701-5713.
2. E. Snell-Rood, Interdisciplinarity: Bring biologists into biomimetics, <https://www.nature.com/news/interdisciplinarity-bring-biologists-into-biomimetics-1.19188>, (accessed 1476-4687 (Electronic)).
3. Asknatureteam, High speed train silently slices through air, <https://asknature.org/idea/shinkansen-train/#.WQvY61WGOUk>, (accessed 03-06-2018, 2018).
4. G. Ingalls, Shinkansen “bullet trains”: Biomimicry at it’s best., <http://blogs.bu.edu/biolocomotion/2011/10/03/shinkansen-%E2%80%9Cbullet-trains%E2%80%9D-biomimicry-at-its-best/>, 2018).
5. E. R. Amelia Hennighausen, 14 Smart Inventions Inspired by Nature: Biomimicry, <https://www.bloomberg.com/news/photo-essays/2015-02-23/14-smart-inventions-inspired-by-nature-biomimicry>, 2019).
6. Y. Tan, S. Hoon, P. A. Guerette, W. Wei, A. Ghadban, C. Hao, A. Miserez and J. H. Waite, *Nat Chem Biol*, 2015, 11, 488-495.
7. A. Miserez and J. H. Waite, 2010, DOI: 10.1036/1097-8542.yb100059.
8. A. Miserez, C. Schneberk T Fau - Sun, F. W. Sun C Fau - Zok, J. H. Zok Fw Fau - Waite and J. H. Waite.
9. D. Ding, P. A. Guerette, S. Hoon, K. W. Kong, T. Cornvik, M. Nilsson, A. Kumar, J. Lescar and A. Miserez, *Biomacromolecules*, 2014, 15, 3278-3289.
10. V. Latza, P. A. Guerette, D. Ding, S. Amini, A. Kumar, I. Schmidt, S. Keating, N. Oxman, J. C. Weaver, P. Fratzl, A. Miserez and A. Masic, *Nat Commun*, 2015, 6, 8313.
11. A. Lipscombe-Southwell, Smart material made from squid teeth a potential plastic alternative, <https://www.sciencefocus.com/news/smart-material-made-from-squid-teeth-a-potential-plastic-alternative/>, (accessed 04/09/2019, 2019).
12. H. Cai, B. Gabryelczyk, M. S. S. Manimekalai, G. Gruber, S. Salentinig and A. Miserez, *Soft Matter*, 2017, 13, 7740-7752.

13. K. Patrick, S. Abbas, Y. Lv, I. Bouelet and X. Zhang, *Pakistan of Journal of Food Sciences*, 2013, 23, 17-25.
14. P. J. Chawda, J. Shi, S. Xue and S. Young Quek, *Food Quality and Safety*, 2017, 1, 302-309.
15. C. BARNARD, Squid beak insight shifts chitosan composites up the pecking order <https://www.chemistryworld.com/news/squid-beak-insight-shifts-chitosan-composites-up-the-pecking-order-/9564.article>, (accessed 04/09/2019, 2019).
16. C. Alvarez-Lorenzo and A. Concheiro, *Chem Commun (Camb)*, 2014, 50, 7743-7765.
17. D. Liu, F. Yang, F. Xiong and N. Gu, *Theranostics*, 2016, 6, 1306-1323.
18. S. C. Sugapriya Dhanasekaran, *Getting a Handle on Smart Drug Delivery Systems – A Comprehensive View of Therapeutic Targeting Strategies*, 2016.
19. K. Petrak, *Drug Discov Today*, 2005, 10, 1667-1673.
20. V. P. T. Bhushan S. Pattni, *Targeted Drug Delivery Systems: Strategies and Challenges*, Springer, 2014.
21. C. Kleinstreuer, E. Childress and A. Kennedy, in *Transport in Biological Media*, eds. S. M. Becker and A. V. Kuznetsov, Elsevier, Boston, 2013, DOI: <https://doi.org/10.1016/B978-0-12-415824-5.00010-2>, pp. 391-416.
22. G. Kaur, J. Grewal, K. Jyoti, U. K. Jain, R. Chandra and J. Madan, in *Drug Targeting and Stimuli Sensitive Drug Delivery Systems*, ed. A. M. Grumezescu, William Andrew Publishing, 2018, DOI: <https://doi.org/10.1016/B978-0-12-813689-8.00015-X>, pp. 567-626.
23. X. Du, T. Zhang, G. Ma, X. Gu, G. Wang and J. Li, *Int J Nanomedicine*, 2019, 14, 2233-2251.
24. Z. W. Lim, Y. Ping and A. Miserez, *Bioconj Chem*, 2018, 29, 2176-2180.
25. N. Tahir, A. Madni, A. Correia, M. Rehman, V. Balasubramanian, M. M. Khan and H. A. Santos, *Int J Nanomedicine*, 2019, 14, 4961-4974.
26. X. Chen, L. Wang, H. Yu, C. Li, J. Feng, F. Haq, A. Khan and R. U. Khan, *J Control Release*, 2018, 288, 173-188.
27. F. Peng, W. Zhang and F. Qiu, *Curr Med Chem*, 2019.

28. W. Zhao, H. Zhang, Q. He, Y. Li, J. Gu, L. Li, H. Li and J. Shi, *Chem Commun (Camb)*, 2011, 47, 9459-9461.
29. L. Zhao, C. Xiao, L. Wang, G. Gai and J. Ding, *Chem Commun (Camb)*, 2016, 52, 7633-7652.
30. H. C. Kim, E. Kim, S. W. Jeong, T. L. Ha, S. I. Park, S. G. Lee, S. J. Lee and S. W. Lee, *Nanoscale*, 2015, 7, 16470-16480.
31. S. Hawkins, M. Wolf, G. Guyard, S. Greenberg and N. Dayan, in *Delivery System Handbook for Personal Care and Cosmetic Products*, ed. M. R. Rosen, William Andrew Publishing, Norwich, NY, 2005, DOI: <https://doi.org/10.1016/B978-081551504-3.50014-6>, pp. 191-213.
32. M. N. Singh, K. S. Hemant, M. Ram and H. G. Shivakumar, *Res Pharm Sci*, 2010, 5, 65-77.
33. W. B. Liechty, D. R. Kryscio, B. V. Slaughter and N. A. Peppas, *Annu Rev Chem Biomol Eng*, 2010, 1, 149-173.
34. C. Englert, J. C. Brendel, T. C. Majdanski, T. Yildirim, S. Schubert, M. Gottschaldt, N. Windhab and U. S. Schubert, *Progress in Polymer Science*, 2018, 87, 107-164.
35. E. W. Neuse, *Met Based Drugs*, 2008, 2008, 469531.
36. K. Leja and G. Lewandowicz, *Polymer Biodegradation and Biodegradable Polymers - a Review*, 2010.
37. G. Dan Mogoşanu, A. Mihai Grumezescu, L. Everard Bejenaru and C. Bejenaru, in *Nanobiomaterials in Drug Delivery*, ed. A. M. Grumezescu, William Andrew Publishing, 2016, DOI: <https://doi.org/10.1016/B978-0-323-42866-8.00008-3>, pp. 229-284.
38. N. Shinde, *Natural Polymers in Drug Delivery Development*, 2014.
39. T. Urbanek, E. Jager, A. Jager and M. Hruby, *Polymers (Basel)*, 2019, 11(6).
40. M. M. G. Carmen Chifiriuc, Alexandru; Grumezescu, Valentina; Bezirtzoglou, Eugenia; Lazar, Veronica; Bolocan, Alexandra, *Biomedical Applications of Natural Polymers for Drug Delivery*, Bentham Science Publishers, 2014.
41. H. Liu, N. Finn and M. Z. Yates, *Langmuir*, 2005, 21, 379-385.
42. S. A. H. Hassani Abdelkader, Norhafizah Abdullah, Suryani Kmaruddin, *Current Research & Reviews*, 2018, 1, 77-84.

43. L. Gasperini, J. F. Mano and R. L. Reis, *J R Soc Interface*, 2014, 11, 20140817.
44. I. Vroman and L. Tighzert, *Materials*, 2009, 2, 307-344.
45. W. Wang, C. Lee, M. Pastuszka, G. W. Laurie and J. A. MacKay, *Pharmaceutics*, 2019, 11(5), 221.
46. J. M. Horn, R. A. Kapelner and A. C. Obermeyer, *Polymers (Basel)*, 2019.
47. S. Sepulveda-Rivas, H. F. Fritz, C. A.-O. Valenzuela, C. A. Santiviago and J. A.-O. Morales.
48. V. Zai-Rose, S. J. West, W. H. Kramer, G. R. Bishop, E. A. Lewis and J. J. Correia, *Biophys J*, 2018, 115, 1431-1444.
49. Y. P. Timilsena, T. O. Akanbi, N. Khalid, B. Adhikari and C. J. Barrow, *Int J Biol Macromol*, 2019, 121, 1276-1286.
50. N. R. Johnson and Y. Wang, *Expert Opin Drug Deliv*, 2014, 11, 1829-1832.
51. W. C. Blocher and S. L. Perry, *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, 2017, 9.
52. G. C. Yeo, F. W. Keeley and A. S. Weiss, *Adv Colloid Interface Sci*, 2011, 167, 94-103.
53. B. Vrhovski, S. Jensen and A. S. Weiss, *Eur J Biochem*, 1997, 250, 92-98.

Chapter 2

Literature Review

In the recent years, increasing attention has been given to develop 'smart' delivery systems for enhanced drug administration while minimizing side effects. 'Smart' delivery systems are notably important for diabetic and cancer patients. In diabetic patients, the amount of insulin needed is difficult to estimate, resulting in accidental dosage errors. Hence, a delivery system that can 'sense' surrounding glucose levels and release insulin accordingly will significantly reduce the risk of incorrect insulin dosage. Similarly, chemotherapeutic drugs are extremely toxic and should only be delivered to tumour site. However, most chemotherapeutic drugs are delivered intravenously resulting in serious side effects. Coacervation has become an increasingly popular method to efficiently encapsulate therapeutics or other molecules. However, only few natural polymers can form coacervates. Recently, DgHBPs have been sequenced and found to exhibit self-coacervation. The first part of the literature review introduces D. Gigas squid beak and coacervation of DgHBPs. The second and third part of the literature review presents insulin and chemotherapeutic drugs delivery systems, respectively.

2.1 Overview of *D. Gigas*

D. Gigas, commonly known as the Humboldt squid, is the largest and most abundant squid in the Ommastrephinae family.¹ They can grow up to 2 meters and weight up to 50 kg. Humboldt squids grow extremely fast during juvenile phases (~5% of its length per day) and reach ~75 cm within 1-year post hatching.² They are found in the East Pacific Ocean and known for their agility and intelligence.³ ³ Humboldt squids are active, aggressive predators that feed on smaller preys such as shrimps, amphipods and various fishes etc.⁴ They are also known to feed on each other if preys are limited. As an aggressive predator, Humboldt squids are armed with two extraordinary biotools, sucker ring teeth (SRT) and squid beak to enhance their predation chances (Figure 2.1).²

Figure 2.1. Illustration of *D. gigas* and its biotools (sucker ring teeth and squid beak).⁵

2.1.1 Sucker ring teeth

Humboldt squids grab their prey using tentacles that are lined with SRT and feed with their sharp beak (Figure 2.2A and B).⁵ SRT are implanted within the sucker cups on all its tentacles and can be recognized by their triangular dentitions. Despite the absence of strengthening phases such as minerals or metals ions, SRT sustain both shearing and compressive stress from predation activities.⁵ SRT are fully proteinaceous with a bias towards 9 amino acids (Ala, Gly, His, Leu, Pro, Ser, Thr, Tyr and Val) and belongs to the suckerins protein family.^{6,7} The top 3 most abundant amino acids are Gly (37 %), Tyr (14%) and His (13%). Sequencing results have shown that suckerins within the SRT are highly modular in nature and strongly resembles silk protein (Figure 2.2C).⁸⁻¹⁰ This modular feature of suckerins is thought to be involved in the formation of β -sheets, which eventually leads to self-assembly of SRT.^{5,6}

Figure 2.2. Features of *D. gigas* SRT. (A) Image of extracted SRT.⁵ (B) Higher magnification of SRT.⁵ (C) Modular architecture of suckerin-19, demonstrating alternating Ala-His and Gly-Tyr rich domains.¹¹

2.1.2 *D. gigas* squid beak and its composition

Other than SRT, the squid beak is the second notable biotool recognized for its remarkable stiffness gradient. The beak is located within the soft buccal tissue and can grows up to ~7 cm (Figure 2.3). The beak, together with its mandibular muscles, act as a feeding tool that is capable of crushing crustacean shells or severing the spinal cord of captured fish.¹² From the results of compositional analysis, the squid beak is found to be a bio-composite biotool comprising 4 main components: chitin, pigments, water and proteins (Figure 2.4).¹³

Figure 2.3. Squid beak of *D. gigas* within the soft buccal tissue.¹⁴

Figure 2.4. Composition analysis of *D. gigas* beak. (A) Protein composition within different regions of squid beak.¹³ (B) Concentration of the 4 main components (chitin, protein, pigments and water) within squid beak by chemical and gravimetric analysis.¹³

Chitin is a polysaccharide made of *N*-acetylglucosamine linked together by β -1,4 linkages.¹⁵ Besides squid beak, chitin is also present within the exoskeletons of many crustaceans and insects.¹⁵ As squid beak is highly crosslinked and sclerotized, it is resistant to most protein extraction reagents, making full protein analysis difficult.^{13, 14} Nevertheless, overall amino acid composition analysis after acid+peroxidation treatment, revealed that Ala and His were the most abundant amino acids within the beak.^{13, 16} The dark colour of the rostral regions within the beaks are insoluble pigments that have been shown by Arnow staining to be crosslinked catechol-rich compounds.^{13, 17, 18} The water percentage in different sections of beak was shown to differ greatly, with the highest percentage (~70%) in the proximal region inside the buccal tissue and lowest percentage (~15%) in the distal region (Figure 2.4B).¹³ Since chitin is found throughout the beak and is hydrophobic, the difference in water percentage appears to be affected by other aspects such as hydrophobicity of beak proteins and amount of pigmentation.

There are visible differences in the level of pigmentation, starting from the translucent colour at the wing edge of the beak to dark brown colour at the tip of the beak (Figure 2.5A to C).¹³ After dissecting and analysing the regions with different pigmentation, it was shown that from the fully pigmented rostrum region to the translucent wing region (Figure 2.5D), the percentage of chitin increased from 15% to 25%, the percentage of water increased from 15% to 75% and the protein content decreased from 60% to 5%.¹³ Interestingly, no mineral phases are present within the beak unlike the hard tissues of most invertebrates and vertebrate such as mollusc shells and bones,¹⁹⁻²¹ indicating that *D. gigas* uses an alternative mechanical strengthening approach than biomineralization for its beak.

Figure 2.5. Pigmentation of *D. gigas* squid beak. (A) lateral view of upper beak without buccal tissue.¹³ (B) Pigment gradient from rostrum to wing of squid beak.¹³ (C) Dissected wing region. (D) sections of beak demonstration different intensity of pigmentation gradient.¹³

2.1.3 Mechanical properties of *D. gigas* beak

The mechanical properties of *D. gigas* beak were measured by tensile testing, fracture toughness assay and nanoindentation (Figure 2.6). Results from these assays demonstrated major differences in the mechanical properties for regions of beak with different pigmentation. At the beak region with most pigmentation (tip of beak), the hardness and Young's modules were ~0.5 GPa and ~5 GPa respectively, which is better than most synthetic polymers (Figure 2.6A).¹² On the other hand, at the proximal region without pigmentation (wing of beak), the Young's modules was only 0.05 GPa (Figure 2.6C).¹² The tip of the beak was predicted to be highly resistance to abrasion. By plotting critical yielding load (H^3/E^2) vs. fracture energy (G_c) of different materials, a graph of predicted abrasion resistance for different materials was obtained (Horizontal line: material failure by plastic deformation, Vertical line: material failure by cracking). In Figure 2.6B, the resistance of squid beak to abrasion was shown to be comparable to dentin, enamel and most metals.^{18, 22} Stiffness between the tip and wing was also found to be ~100-fold

different. This stiffness gradient within the beak was thought to reduce contact injury and mechanical disparity between the soft buccal tissue and hard beak. Similarly, tensile assays also showed a mechanical gradient between the tip and wing region (Figure 2.6C and D). Interestingly, beak regions with different pigmentation level after freeze-dried displayed similar Young's modulus and hardness, confirming that stiffness and hardness were closely correlated to hydration levels of beak tissue.

Figure 2.6. Mechanical characterisation of *D. gigas* beak. (A) Ashby plot (Young's modulus (E) vs Hardness (H)) of squid beak with common materials.¹² (B) Critical yielding load (H^3/E^2) vs fracture energy (G_c) of different materials.¹² Horizontal and vertical lines were critical loads for yielding and cracking respectively. (C) Young's modulus and tensile strength in different areas of squid beak.^{13, 16} (D) Tensile stress vs tensile strain curves for different regions of hydrated beak.¹³

Since mechanical and composition gradient are both present within the beak, these data suggest that the mechanical properties e.g. stiffness gradient and hardness can be related to the changes in composition (protein, chitin, water and pigments) gradient. While the exact mechanism is still currently unknown, it has been hypothesised that beak maturation was due to dehydration of chitin fibers and inter-crosslinking of beak proteins.¹⁸

The mechanical properties of chitin fibers are strongly dependent on its hydration state, with elastic modules of dehydrated chitin and hydrated chitin to be ~40 GPa and 15 MPa respectively.^{23, 24} Likewise, the compactness of the chitin scaffold can be affected by its water content, leading to differences in the stiffness.¹⁶ Hence, the mechanical properties of the squid beak appeared to be adjusted by controlling the level of hydration within the chitin scaffold. As chitin is normally hydrophobic, its wettability must be facilitated by other beak components such as beak proteins or pigments.

While the dehydration mechanism of chitin fibers is still being debated,^{18, 25} one common hypothesis is the participation of beak proteins to control the porosity of chitin scaffold. The crosslinking of beak proteins through aromatic residues leads to the pigments production, decreasing the porosity of chitin scaffold in the process and ultimately increasing the stiffness of the beak. Another possible mechanism is the infiltration of hydrophobic beak proteins into the chitin scaffold, which eventually leads to chitin dehydration. Nucleophilic additive reactions between the imidazole of His residues and catechol rings was confirmed by NMR and MS/MS.¹⁸ The origin of catechol rings is currently unclear. It can be either secreted by beccublast cells (low molecular weight catechol molecules) or by post-translation modification of Tyrosine into 1-3,4-dihydroxyphenylalanine (L-DOPA), a catechol containing unnatural amino acid.^{18, 26} Indeed, L-DOPA had been identified during the amino acid analysis of squid beak.¹³ Nonetheless, more research is needed to identify the exact source of catechol rings.

2.1.4 Inspired materials from *D. gigas* squid beak

The unique composition and mechanical gradient of *D. gigas* squid beak have offered inspiration for synthetic materials. Two different squid beak-inspired composite materials will be described next. In Figure 2.7, a chitosan/ γ -Fe₂O₃ composite film was fabricated by following squid beak sclerotization model.²⁷ The sclerotization of squid beak was thought to result from both the dehydration of chitin fibers and imidazole-catechol crosslinking.

Figure 2.7. Illustration of squid beak-inspired, catechol-functionalised chitosan+ γ -Fe₂O₃ composite film.²⁷

In this composite film, catechol moieties were first added to a chitosan scaffold with the purpose of increasing the hydrophobicity (by replacing amine groups) and allowing subsequent crosslinking (coordination or covalent crosslinking of catechol moieties). γ -Fe₂O₃ nanoparticles were subsequently added to trigger catechol-Fe coordination crosslinking.²⁷ Therefore, the chitosan scaffold in this composite film emulates the chitin scaffold while the catechol-Fe coordination bonds emulate the imidazole-catechol bonds within the *D. gigas* squid beak. The final composite film demonstrated high stiffness (~1 GPa), further proving the role of chitin dehydration and protein crosslinking on improving mechanical properties (Figure 2.8A).²⁷ In addition to coordination crosslinking, γ -Fe₂O₃ nanoparticles also impart magnetic properties which can be useful in different medical applications (Figure 2.8B and C).²⁷

Figure 2.8. Characterisation of chitosan+ γ -Fe₂O₃ composite film. (A) Young's modules of hydrated and dehydrated film. **(B)** Magnetisation curve for chitosan+ γ -Fe₂O₃ composite film. **(C)** Experimental setup for magnetisation studies of chitosan+ γ -Fe₂O₃ film.²⁷

In another example, a composite film based from poly-vinyl acetate (PVAc) was also inspired by the molecular gradient of *D. gigas* beak (Figure 2.9A). In this composite

film, PVAc matrix acted as the chitin scaffold and alkene-modified cellulose nanocrystals (Allyl-CNCs) mimicked the protein matrix (Figure 2.9B).²⁸ Different regions of composite films were then exposed to different period of UV to trigger a differential gradient of thiol-ene crosslinking. This crosslinking gradient mimicked the sclerotization gradient found in squid beak.²⁸ The final film demonstrated a mechanical gradient that was dependent on the hydration state similar to the squid beak (Figure 2.10A and B). In contrast, composite films that were uniformly crosslinked did not exhibit the mechanical gradient seen in films with gradient crosslinking (Figure 2.10C).²⁸

Figure 2.9. Squid beak-inspired allyl-CNCs+PVAs composite film.²⁸ (A) Illustration of the film casting and photo-crosslinking procedures. (B) Demonstration of hydration-enhanced mechanical gradient model in squid beak and squid-inspired composite film.

Figure 2.10. Mechanical properties of composite film.²⁸ (A) Tensile storage modulus of uncrosslinked and crosslinked composite film in either dry or wet conditions. (B) Illustration of Allyl-CNC+PVAc composite film. Solid lines represent regions with increasing amount of UV exposure whereas red lines represent regions of films that were cut out for mechanical testing. Experiments were performed on samples that were wet at 37 °C, dry at 25 °C and dry at 80 °C (C) Twisting of composite film with gradient crosslinked film (top panel) and uniformly crosslinked gradient (bottom panel).

2.1.5 Extraction and identification of *D. giga* beak proteins (DgBPs)

To further understand the relationship between the molecular and mechanical gradient within the squid beak, it is essential to sequence and characterise the proteins found in beak and along with understanding their post secretion processes. Squid beak proved extremely challenging to sequence due to the difficulties in protein extraction from the heavily crosslinked beak. Conventional protein extraction methods are not useful for highly crosslinked tissues such as squid beak as most of the proteins remained insoluble even with the most destructive extraction protocols. Hence, a new method was used where beak proteins were cleaved chemically by peptide bond cleaving reagents such as hydroxylamine and formic acid. The beak proteins were then released from the beak in the form of peptide and proteins fragments.^{14, 29, 30} These peptide and protein fragments were then subjected to different protein detection assays, i.e. nitroblue tetrazolium staining (NBT) for detection of catechols, Pauly's staining for detection of His-rich proteins, lectin binding

assay for detection of chitin oligomers and SDS-PAGE for information on size of protein fragments (Figure 2.11).^{14, 31} These assays allowed for simple and fast confirmation of different possible functional groups within these protein fragments, and could be done without any prior knowledge on the protein sequence. These detection assays confirmed that the fragment size ranged from 5 to 250 kDa, histidine-rich proteins were present within the fragments and chitin oligomers exist within the larger molecular weight fractions.¹⁴ In gel digestion followed by tandem mass spectrometry and Edman sequencing were then used to identify the partial sequence of these extracted proteins.¹⁴

Despite the above methods, only partial sequences of the beak proteins were initially identified. To further identify the remaining of the sequences, RNA sequencing was used to generate a transcriptome library, extracting mRNA from the buccal tissue where the beak proteins are secreted.¹⁴ Buccal tissue was chosen for the transcriptome library because it contains beccublast cells, which were involved in the expression of beak proteins. A *de novo* assembly method by trinity software package was subsequently used to assemble the mRNA sequences.¹⁴ Full-length beak protein sequences were then identified by alignment of partial sequence to the transcript sequence and by matching to the amino acid profiles. Rapid amplification of cDNA ends (RACE)-PCR and sanger sequencing was finally used to confirm the full-length protein sequences.^{14, 32, 33}

Figure 2.11. SDS PAGE of extracted beak proteins with different staining.¹⁴ Coomassie Blue R-250 (CBR) staining for all proteins; Pauly staining for His-rich proteins; NBT staining for proteins containing catechols and Lectin binding assay to identified *N*-Acetylglucosamine oligomers. (L= protein ladder)

From the results of the beak protein sequencing, two families of beak proteins: *D. gigas* chitin binding beak proteins (DgCBPs) and *D. gigas* histidine-rich beak proteins (DgHBPs) were identified (Figure 2.12).¹⁴ There are four variants of DgCBPs and each of them contains at least two chitin-binding domains similar to peritrophin-44, a common chitin-binding protein (Table 2.1).^{14, 34} Therefore, DgCBPs is thought to act as a chitin binding protein within the chitin scaffold of squid beak. From the protein fragments sequencing, there is another group of proteins with high percentage of His, Ala and Gly, found only in the tanned regions of the beak.¹⁴ After screening the transcriptome library for proteins with His, Ala and Gly bias, three different variants were found and named DgHBPs.¹⁴ Since this project uses DgHBPs and its coacervation properties for fabrication of drug delivery system, the next section will discuss DgHBP sequences in more details.

Figure 2.12. Illustration of DgCBP and DgHBP protein family.¹⁴ X represents a hydrophobic residue, whereas X' is normal Tyr and X'' is generally either Gly or Ala.

Table 2.1. Sequences of DgCBPs and techniques used to identify them.¹⁴

Proteins	Techniques used	cDNA Deduced Sequence (3' RACE verified)	Predicted Mass / pI
DgCBP-1	MS/MS Transcriptome search	<u>MG</u> TNGLPNVG <u>LIL</u> FLWGLTF <u>CVA</u> FGQSFDI <u>CAG</u> GLTGVFE <u>DK</u> TDRCNLF <u>CL</u> SGRLKHFR <u>CH</u> NNKVFSK <u>TK</u> TCVTLNEA <u>AG</u> QCSTDFSR <u>KV</u> HEIVNPYS <u>YC</u> QLHPTAVL <u>PH</u> PDSCALFY <u>NC</u> SRPTSLGK <u>YF</u> SPYVDECP <u>Y</u> PELFSKTTK <u>R</u> CEPYRTVHC <u>ER</u> RPEPRDVC <u>QY</u> RQNWCGQS <u>HC</u> VPCHIRFP <u>SC</u> RGKSEGLN <u>SV</u> PYVKWGPQ <u>YI</u> ECRHGRLI <u>TK</u> SKCIGKPG <u>QP</u> AVFSPQSR <u>SC</u> ISVFSVSN <u>P</u> PTNPISCRY <u>K</u> SDGFYANPT <u>N</u> CKKYFQCLN <u>QN</u> FIDMLECP <u>EN</u> LVFDSRAA <u>SC</u> VPSNSCP <u>PC</u> GTKIGC	33.1 kDa / 8.9
DgCBP-2	MS/MS Transcriptome search	<u>MY</u> R TAA I F IV <u>VIV</u> GVVICQD <u>NL</u> ISICKNVK <u>RR</u> VGTNLVRD <u>QG</u> NCSVFYSC <u>RH</u> PNRPFPM <u>AC</u> GYTTVFSQ <u>SQ</u> QVCVMQNS <u>IY</u> DDCKRSIY <u>GG</u> TANDPLCR <u>PH</u> PSGLNRDP <u>AD</u> CTRFIPCF <u>NH</u> TLILHVCQ <u>DG</u> SLHLLSKE <u>CC</u> RTGDLMPH <u>NS</u> HTHTF	15.8 kDa / 8.3
DgCBP-3	Edman Transcriptome search	<u>MY</u> IKALIVIG <u>LV</u> SVLAGLAT <u>NA</u> FT CPKGT <u>SW</u> IRDPNCS <u>LN</u> HWCI NG NY <u>SG</u> SFYCGPGY <u>V</u> AGGSAALCM <u>V</u> APQYDWN <u>CK</u> DLMKINIE <u>ND</u> PRCAH NG <u>SN</u> PEPTDCAR <u>F</u> VECWQGTSM <u>SV</u> KWC PKGLL <u>YN</u> ATKRICMW <u>PT</u> RVNANRP <u>N</u> KMADSAKIS <u>K</u> FRTECNTAR <u>GK</u> GHMLIAYP <u>GD</u> CSQYISCD <u>SN</u> DQSPQOCA <u>SG</u> TVFNSEKQ <u>RC</u> DFRANVPS <u>CK</u> VPA	22.6 kDa / 8.7
DgCBP-4	Transcriptome search	<u>M</u> FLYATCVFL <u>A</u> AGLVLSTAQ <u>I</u> ATNPCEVAG <u>V</u> HTQLMADPT <u>SC</u> SNFFFCVG <u>KI</u> PRMECGP <u>GT</u> LFNARIKM <u>CD</u> HAENVNCD <u>DT</u> KRPIVPPT <u>K</u> SPSVRPTV <u>PI</u> HDTKLVEQ <u>Y</u> YERWYK F YC <u>SE</u> YGSNNVVR <u>W</u> PHPNKCDRF <u>YE</u> CRQQLSA <u>V</u> VCPTYPQOM <u>LY</u> NRTLQNC <u>LP</u> QVRCEKS <u>LP</u> FGNNFASA <u>SE</u> EELKYRAL <u>CK</u> PGTMSFNR <u>WA</u> NSHDCRRF <u>Y</u> QCLN	23.9 kDa / 8.8

2.1.6 Sequence features and primary structure analysis of DgHBPs

The sequences of DgHBPs variants were confirmed using RACE-PCR (Table 2.2).¹⁴ These DgHBP sequences were aligned using online protein databases (SwissProt and GenBank) and no other homologous proteins were found, suggesting that they are a novel class of proteins with an unique gene family. All DgHBPs are highly conserved and organised into two main domains, one of which (the C-terminus) consists of extensive modular architecture.¹⁴ The N-terminal domain of these proteins contains non-repetitive, long stretches of Ala- and His-rich regions whereas the C-terminal domain consists of mostly tandem His- and Gly-rich repeats.¹⁴ Among the 3 variants, only DgHBP-1 and DgHBP-2 have been successfully expressed and therefore a detail comparison of DgHBP-1 and 2 will be described next.

Table 2.2. Protein sequence of DgHBP-1, 2 and 3, with the repeat domain sequences underlined.¹⁴

Protein	Techniques Used	cDNA Deduced Sequence (3' RACE verified)	Predicted Mass / pI
DgHBP-1	Amino acid composition (transcriptome search), MS/MS	<p> MKCVSFAIFL VVAIFGANSQ LYGAPAVGGV VENAVNAAES GAAATHDAQG AYAEADTAGV LDVNHAEHHD GVHDASGYGF GGLAGHGGFA GHGLYGPFGA GHGLLGAGYA GLGLHGAGFA GHGLHGAGFA GHGLYGAGFA GHGLHGFAFH GLYGAGFAFH GLGLGGLHGA LGHGALAHY </p>	14.9 kDa / 6.0
DgHBP-2	Amino acid composition (transcriptome search)	<p> MKFCLSVALL AVAMVMVNGQ FFGAGPFNTA HHSVSDAAA AHHDAAGEYA QNAATGLLDT HHNENHDMTH DLANGYGLHE HDEQHHGLAD GLHQEYAARA AQGANAVHND AAQSHSALAA ANTFGHGHP YAAIGHGVY HGPYGHGPY HGLYGHGLY HGPYGHGLY HGAFGHGLNA YAPLVGHGLR GYL </p>	16.7 kDa / 6.1
DgHBP-3	Amino acid composition (transcriptome search), MS/MS	<p> MNCYSFAIFL AVAILGARSQ YYGGVGGGLG GLHGLAGGVG GLHGLAGGVG GFAGSSGFHE HDEEHGVD GVHQAYATHA AQGAHAHDA AQRHSAALAA ASAAHGGLAG FGGLAGHGAG FAGHGLGLAG HGFAGHGLHG AGFAGHGLGL AGHGFAGHGL HGAGFAGHGF GLGGHGLYGH Y </p>	15.1 kDa / 6.5

Both DgHBP-1 and 2 have weakly acidic isoelectric point (pI) (~ 6), but most of the acidic residues (Glu and Asp) are found only on the N-terminal region (Table 2.2). Ala-rich clusters are also found in the N-terminal region of both proteins. In contrast, the basic residues (His for DgHBP-1, Arg and His for DgHBP-2) are found throughout the sequence.¹⁴ These residues are found in His-rich clusters in the N-terminal region where they are frequently surrounded by Gly residues and at part of the tandem repeats in the C-terminal region. More than 60% of the protein sequences in DgHBP-1 and 2 were found to be more hydrophobic than Gly.¹⁴ The molecular weight (Mw), amino acid number (A. A.), pI, and acidic/basic/aromatic residues count ($R^{\text{acidic}}/R^{\text{basic}}/R^{\text{arom}}$) for DgHBPs have been summarized on table 2.3.

A common feature of both proteins is the tandem His-, Tyr- and Gly-rich pentapeptides located at the C-terminal region (Table 2.2, underlined sequences). Within these repeats, His residues are always surrounded by two Gly residues while Tyr residues are frequently located on the fifth position.¹⁴ Of the pentapeptides, GHGLY and GHGPY are the most common motifs with differences on the 4th and 5th positions (Table 2.4). These

repeats share similarity with the Gly- and Ala-rich tandem repeats located within the hydrophobic domain of tropoelastin, which have proven to be important for elastin coacervation.¹⁴ On the other hand, a unique feature of DgHBPs pentapeptides is the high percentage of His residue, suggesting that pH has an important role in the control of coacervation given that the pKa of His is around pH 6.5. DgHBP-1 has an additional pentapeptide motif (GAGFA) that is completely absent in DgHBP-2.¹⁴

Table 2.3. Molecular weight (Mw), amino acid number (A. A.), pI, and acidic/basic/aromatic residues count ($R^{\text{acidic}}/R^{\text{basic}}/R^{\text{arom}}$) for DgHBPs.³⁵

Protein	MW (Da)	A.A.	pI	R^{acidic}	R^{basic}	R^{aromatic}
DgHBP1	15186	161	6.03	9	19	16
DgHBP2	18195	175	6.15	13	29	19

Table 2.4. Tandem repeats within the C-terminal of DgHBPs. Underlined sequences represent conserved sequences within the repeats.¹⁴

DgHBP-1			DgHBP-2			DgHBP-3		
85	GHG - - - GFA	90	125	GHGHA PYAAY	134	30	GGLHGLAGGV	39
91	GHGLYGPFA	100	135	GHGVY	139	40	GGLHGLAGGV	49
101	GHGLLGAGYA	110	140	GHGPY	144	50	GG	51
111	GLGLHGAGFA	120	145	GHGPY	149	Consensus	GGLHGLAGGV	
121	GHGLHGAGFA	130	150	GHGLY	154			
131	GHGLYGAGFA	140	155	GHGLY	159		107	GLA - - - GF - G
141	GHGLH - - GFA	148	160	GHGPY	164	113	GLAGHGAGFAGHGL	126
149	GHGLYGAGFA	158	165	GHGLY	169	127	GLAGH - - GFAGHGL	138
159	GHGL - GFG	GL 167	170	GHGAF	174	139	- - - HGAGFAGHGL	148
Consensus	GHGLYGAGFA		175	GHGLN AYAPLV	185	149	GLAGH - - GFAGHGL	160
			186	GHGLR GYL	193	161	- - - HGAGFAGHGF	170
			Consensus GHGLY			171	GLGGH - - GLYGH - Y	181
168	HGALG	172				Consensus	GLAGHGAGFAGHGL	
173	HGALA	177						
178	H	178						
Consensus	HGALG							

2.1.7 Secondary structure characterisation of DgHBP-1 and 2

CD spectroscopy have been used to characterise the secondary structure of DgHBP-1 and -2 in solution (10 mM acetic acid, pH 3.3). DgHBPs were prepared in 10 mM acetic acid as they were most soluble in this solvent and would not undergo coacervation at this pH. The obtained CD spectrum demonstrated a concentration dependent conformation change in both proteins (Figure 2.13).³⁶ At low concentrations (1 mg/mL), both proteins exhibit a spectrum that is indicative of a disordered structure. As the concentrations

increases to 10 mg/mL, a shift from disordered structure to β -structures (β -turns and β -sheets) was observed.³⁶ This concentration-dependent conformation change was also seen in α -elastin.³⁷ Although this negative peak was already present in DgHBP-1 at low concentration, the peak became more obvious at higher concentrations (Figure 2.13A).³⁶ Since only DgHBP-1 also contains GAGFA/GFA motifs and displays β -structures at low concentration, this suggests that GAGFA/GFA motifs may form β -structures. From the spectrum, DgHBP-2 could also exhibit polyproline II conformation, which is facilitated by its high percentage of Ala and Leu (Figure 2.13B).^{38, 39} In contrast, DgHBP-2 contains Pro-Gly β -turns that are similar to those found in elastin-like poly-pentapeptides.⁴⁰⁻⁴² Indeed, only DgHBP-2 has Pro-containing tandem repeats (GHGPY) that are not seen in DgHBP-1. The percentage of secondary structures within the DgHBPs were analysed using solid state Fourier Transform Infrared Spectroscopy (FTIR) and summarised on Table 2.5. The dominant secondary structure in DgHBPs were β -sheets, with DgHBP-2 having a higher β -turn content (32%) when compared to DgHBP-1 (16%). The higher β -turn in DgHBP-2 may be due to its higher Pro content as Pro is known to be responsible for the formation of β -turns in tropoelastin studies.⁴³

Figure 2.13. CD spectrum of (A) DgHBP-1 and (B) DgHBP-2 at low (1 mg/mL) and high concentration (10 mg/mL).³⁶

Table 2.5. Percentage of different secondary structures in DgHBPs obtained by FTIR analysis.³⁵

Protein/secondary structure	β -sheets (%)	α -helix (%)	β -turn (%)
DgHBP-1	62.2	7.3	16.2
DgHBP-2	53.6	14.5	31.9

Since the coacervation of tropoelastin can be triggered by heating (Lower Critical Solution Temperature, LCST phase transition), DgHBP-1 and 2 solutions (in 10 mM acetic acid) were heated to investigate if a similar phase transition can be observed (Figure 2.14). In contrast to tropoelastin, heating the protein solution did not result in coacervation³⁶ However, significant changes in the CD spectra of both heated proteins were seen. From Figure 2.14, the protein structures are shifting from random coil structure to a more ordered structure (β -structures). Together, these data suggest that as the temperature increased, β -structures were formed by inter-molecular hydrophobic interactions between the protein molecules. Unlike tropoelastin, these hydrophobic interactions were insufficient to induce coacervation in DgHBPs as coacervation of DgHBPs was not observed by microscopy after heating.^{35, 36}

Figure 2.14. CD spectrum of (A) DgHBP-1 and (B) DgHBP-2 at different temperatures.³⁶

2.1.8 Coacervation conditions of DgHBPs

Both DgHBPs under simple coacervation when added into their respective buffers. Turbidimetry was used to determine the optimum conditions for DgHBPs coacervation. At 100 mM ionic strength, both proteins reached optimum coacervation within a small pH range (pH 6.5 for DgHBP-1 and pH 6.5 to 7.0 for DgHBP-2) (Figure 2.15).^{36 35} Both proteins' optimum coacervation pH were slightly above their pI (DgHBP-1 pI = 6.03, DgHBP pI = 6.15). The pH for maximum coacervation in both proteins is near to the pKa value of His, indicating that intermolecular interactions of DgHBPS are closely related to the de-protonation of His residues. At acidic conditions, DgHBPs are positively charged resulting in strong electrostatic repulsions between the protein molecules, preventing them from coacervation. At pH near to pI of DgHBPs, strong electrostatic repulsions disappear, allowing weak attraction forces such as hydrophobic and coulombic interactions to dominate and ultimately coacervation to take place. At higher salt concentrations (1 M ionic strength), the coacervation of DgHBPs occurred over a wider range of pH (DgHBP-1 = pH 4.0 to 7.5, DgHBP-2 = 4.0 to 8.0) (Figure 2.15).³⁶ This wider range of coacervation pHs is due to charge screening of His residues at higher salt concentrations, which prevents the strong electrostatic repulsions and favours the weaker attraction forces.

Figure 2.15. Relative turbidity of (A) DgHBP-1 and (B) DgHBP-2 coacervates in different buffers (ionic strength and pH). NaCl was used to adjust the ionic strength of the buffers.³⁶

Although both DgHBPs undergo coacervation, their dynamics and morphology of coacervation are different. DgHBP-1 formed a gel-like condensed phase (condensed

coacervates) and maintained its consistency until it was disturbed by pipetting.³⁶ Upon disturbance, the condensed coacervates dispersed and the solution become homogenous turbid, also known as dispersed coacervates (Figure 2.16, Left column). In contrast, DgHBP-2 coacervates did not form this gel-like layer and diffused into the buffer quickly as dispersed coacervates (Figure 2.16, Right column).³⁶ Intentional disturbance by pipetting only increased the rate of coacervates diffusion.

Figure 2.16. Optical micrographs of DgHBP-1 (left column) and DgHBP-2 (right column) coacervates over time.³⁶

While DgHBPs coacervates coalescence over time, the merging kinetics were different for the two proteins (Figure 2.17). The initial size of DgHBPs coacervates were $\sim 1 \mu\text{m}$. However, the rate of coalescence was slower for DgHBP-1 (Figure 2.17, top panel) when compared to DgHBP-2 (Figure 2.17, bottom panel).³⁶ This difference in merging kinetics suggests that DgHBP-1 coacervates is more stable against coalescence than DgHBP2.

Figure 2.17. DLS measurement of DgHBP-1 (top panel) and DgHBP-2 (bottom panel) coacervates over time.³⁶

Also, only DgHBP-1 was found to display shear-thinning behaviour (100-times reduction in viscosity) below of critical shear rate ($\sim 15/\text{s}$) (Figure 2.18A). The initial viscosity of DgHBP-2 was already much lower than DgHBP-1 and did not showed any obvious shear-thinning behaviour. Frequency sweep measurements (0.1 to 10 Hz) by rheology showed that DgHBPs displayed a gel-like behaviour where the storage modulus (G') was higher than viscous modulus (G'') (Figure 2.18B).³⁶ Since the G' of DgHBP-1 was two times higher than DgHBP-2, this suggested that DgHBP-1 coacervates are more

compact. The high G' of DgHBP-1 may be due to the presence of GAGFA/GFA motifs that are absent in DgHBP-2.³⁶ To test out this hypothesis, consensus peptides containing C-terminal motifs of DgHBPs (pepHBP-1: GHGLY GAGFA GHGLH GFA GHGLY and pepHBP-2: GHGVY GHGVY GHGPY GHGPY GHGLY W) were designed and compared with rheology. Like DgHBP-1, pepHBP-1 also displayed significant higher G' than pepHBP-2, suggesting that the G' difference is due to GAGFA/GFA motifs (Figure 2.18C and D).³⁶

Figure 2.18. Rheology results of DgHBPs and its consensus peptides.^{14, 36} (A) Viscosity of DgHBPs vs shear rate. (B) Storage (G') and viscous(G'') modulus of DgHBPs. (C) Viscosity of DgHBPs consensus peptides (pepHBP-1 and pepHBP-2) vs shear rate. (D) Storage (G') and viscous(G'') modulus of pepHBPs vs shear rate.

2.1.9 Time-dependent structural changes of DgHBPs coacervates

CD spectroscopy was used to monitor structural changes of DgHBP coacervates over time (Figure 2.19). The secondary structures of DgHBPs in coacervates state were

dynamic and changed over time. The CD spectrum of DgHBP-1 indicate that the β -sheets were formed over time in the coacervates.³⁶ The intensity of the negative peak at 208 nm was reduced and shifted over time in DgHBP-2 (Figure 2.19A).³⁶ In contrast to DgHBP-1, β -sheets were not formed within DgHBP-2 coacervates (Figure 2.19B).³⁶ The CD spectrum of DgHBP-2 also indicates that Pro-Gly β -turns were formed over time.

Figure 2.19. CD spectrum of (A) DgHBP-1 and (B) DgHBP-2 coacervates over time.^{14, 36}

2.1.10 Coacervation of DgHBP-derived peptides

A consensus peptide (DgHBP peptide: GHGVY GHGVY GHGPY GHGPY GHGLY) derived from the tandem repeats of DgHBP-2 protein is able to undergo simple coacervation.¹⁴ This peptide is 25 amino acids long, consisting of five GHGXY repeats. From the turbidity results, optimal coacervation of DgHBP peptide occurs at pH 8 with 0.5 M ionic strength (Figure 2.20A). These conditions are very similar to salinity and pH of seawater. Like DgHBP-2, DgHBP peptide coacervates did not form gel-like phase and dispersed immediately into the buffer to form dispersed coacervates (Figure 2.20B).¹⁴

Figure 2.20. Coacervation of DgHBP peptide in phosphate buffer.¹⁴ (A) Relative turbidity at different pH and ionic strength. (B) Light micrograph of DgHBP peptide coacervates. ($n = 3$, technical replicates, error bars represent mean $\pm$ s.d.)

Similarly, a consensus peptide from DgHBP-1 (PepHBP-1: GHGLY GAGFA GHGLH GFA GHGLY) is also able to undergo simple coacervation at its respective buffer conditions.³⁶ However, turbidimetry results for this peptide have not been reported.

2.2 Overview of diabetes and glucose responsive insulin delivery system (GRIDS)

Diabetes is a chronic medical condition characterised by high fasting blood glucose levels. Both type I and late type II diabetes require the administration of insulin for controlling blood glucose level. For these patients to attain good therapeutic outcomes, a stringent insulin replacement program is required.⁴⁴ Despite knowing the importance of glycaemic control, many patients still suffer from complications due to inadequate blood glucose control or insulin administration. Therefore, closed-loop insulin delivery methods are now being actively investigated to overcome these problems.⁴⁴⁻⁴⁷

A closed-loop delivery method can sense glucose level while delivering the required amount of insulin in different situations.^{46, 48} This reduces the patient's direct participation in glucose management and prevents insulin over/under dosage. An ideal delivery system can respond rapidly and stops immediately once the glucose level reaches normal level to prevent insulin overdose (Figure 2.21). Currently, the most advance development of a close-looped system is by integrating both digital insulin pumps and glucose sensors into an automated control device. However, this device is less attractive to some patients as it is relatively expensive and bulky.

The other approach for a closed-looped delivery system is to develop smart materials that can sense glucose. These smart material systems are classified into three different mechanisms: enzymatic, diol-binding chemistry, and glucose-binding proteins.⁴⁵ Enzymatic and diol-binding chemistry mechanisms will be the focus of this literature review since they are commonly integrated in closed-looped delivery systems.

Figure 2.21. Illustration of a closed-loop insulin delivery method.⁴⁹ This delivery system is capable of sensing the glucose level while releasing the correct amount of insulin at the same time. Once the glucose level goes back to normal, insulin release will stop.

2.2.1 Glucose sensing by enzymatic method

Out of the different enzymes that can recognise glucose, GOx, is most widely used in the development of smart material systems. GOx catalyses the conversion of β -D glucose into D-glucono- δ -lactone and hydrogen peroxide (H_2O_2) (Figure 2.22). D-glucono- δ -

lactone will then be further hydrolysed into gluconic acid, which results in a pH decrease. The optimum pH range for conversion of glucose to gluconic acid is between 5.0 to 7.0. The pH decreases (down to pH 4.0) with increasing concentration of gluconic acid and the rate of glucose conversion decreases when the pH falls outside the optimum range.^{50, 51} In these ‘smart’ insulin delivery systems, a localised decrease in pH from production of gluconic acid are used to initiate conformational or structural changes, which eventually lead to release of insulin.⁴⁹

Figure 2.22. Chemical reaction of glucose to gluconic acid catalysed by GOx.⁵² GOx oxidises glucose into D-glucono- δ -lactone, which further hydrolyses to gluconic acid.

Conformational or structural changes of these smart materials can lead to changes in their permeability. This increase in permeability can be due to swelling of the smart materials. Gu and co-workers have shown that glucose-responsive chitosan microgels can be made by encapsulating Insulin and GOx nanocapsules.⁵³ GOx was encapsulated in acrylamide-based nanocapsules to increase its stability. When glucose level increased, GOx converted more glucose into gluconic acid resulting in a decrease of pH within the microgel. The subsequent protonation of the chitosan chains resulted in repulsion and microgel swelling (Figure 2.23A).⁵³ Insulin was then released because of changes in permeability due to microgel swelling. Since glucose is a ubiquitous molecule, an ideal responsive system should be able to differentiate high blood glucose level from normal fasting glucose level to prevent insulin overdose. Only high glucose concentration (400 mg/dL) but not low glucose concentration (100 mg/dL) resulted in the release of insulin from the microgel (Figure 2.23B).⁵³

Figure 2.23. Glucose-responsive microgel loaded with GOx and Insulin.⁵³ (A) Illustration of a glucose-responsive microgel loaded with both GOx and Insulin. GOx converts glucose into gluconic acid resulting in a localised decrease pH. The subsequent protonation results in microgel swelling and release of insulin. (B) Insulin is only released in the presence of high glucose concentration (400 mg/dL) but not in low glucose concentration (100 mg/dL) or PBS.

Decrease in localised pH from gluconic acid can also lead to shrinking of smart materials. Chu and co-workers have synthesised a glucose-responsive polyvinylidene fluoride (PVDF) membrane through the additional of a glucose sensing gating membrane onto the pores (Figure 2.24).⁵⁴ The gating membrane was made of immobilised GOx on poly(acrylic acid) (PAAC) membrane. At physiological pH/low glucose level, carboxyl groups on the PAAC chains remained negatively charged.⁵⁴ The PAAC chains dissociated due to charge repulsion and pores of PVDF membrane closes. In the presence of glucose, GOx converted glucose to gluconic acid, thereby locally decreasing the pH at the pore of

the PVDF membrane. The subsequent protonation of carboxyl groups decreased electrostatic repulsion and pores of PVDF membrane opened.⁵⁴

Figure 2.24. Illustration of glucose-responsive gating membrane on a porous PVDF membrane substrate.⁵⁴ GOx converts glucose to gluconic acid and decreases the local pH. The protonation of the carboxyl groups results in the shrinking of PAAC chains and thus to the opening of PVDF membrane pores.

Another mechanism of insulin release by GOx sensing is the use of acid-labile materials. These materials degrade faster at pH lower than physiological pH, allowing insulin to be released. Acid labile polymers such as polyesters or acetyl-modified dextran have been used to develop GOx-based responsive delivery systems.⁴⁵ Tai and co-workers have created a glucose-responsive polymersome-based nanovesicle that dissociates at low pH (Figure 2.25A).⁵⁵ The nanovesicle is made of poly(ethylene glycol) (PEG) and ketal-modified polyserine (Ser-Ketal). When glucose is converted into gluconic acid, the aqueous core of nanovesicle becomes acidified. This acidification hydrolyses the acid labile ketal groups, resulting in the formation of water-soluble PEG-polyserine and led to dissociation of the nanovesicles (Figure 2.25B).⁵⁵

Figure 2.25. Illustration of glucose-responsive nanovesicle loaded with Insulin, GOx and catalase.⁵⁵ (A) Conversion of glucose to gluconic acid acidifies inner core of nanovesicle, resulting in dissociation and release of its contents. (B) Chemical structure of Ser-Ketal and its hydrolysis into PEG-polyserine at low pH.

2.2.2 Diol-binding chemicals

Synthetic molecules containing the boronic acid functional group, such as phenylboronic acid (PBAs), can bind reversibly to diols such as glucose.⁵⁶⁻⁵⁹ This reaction occurs at basic pH, leading to the formation of boronate ester. Depending on the pH, either neutral or anionic esters can be formed (Figure 2.26).⁵⁶ Once formed, anionic boronate esters are more stable than neutral boronate esters. However, these esters are unstable under acidic conditions and completely dissociate at lower pH. The stability of the boronate esters also depends on the type of diols involved. Boronate esters from *Cis*-1,2 diols have the highest relative affinity, followed by *Cis*-1,3 diols and *trans*-1,2 diols.⁵⁶

Figure 2.26. Formation of boronate esters from PBA and diols at different pH.⁵⁶

One mechanism of using PBA to control insulin release is through charge repulsion to induce swelling in a hydrogel. When PBA is bound to glucose, the anionic boronate esters are more stable than the neutral free PBA (Figure 2.27).^{56, 60} As the negative charges accumulate, the hydrogel starts to swell, and insulin will be released.

Figure 2.27. Anionic boronate ester formed between PBA and glucose in aqueous solution.⁶⁰

PBA can be used as a cross-linker to stabilise materials loaded with insulin.⁶¹ Polymers imprinted with glucose or other diols molecules can be used to form nanoparticles or hydrogels. These diols act as an anchor point for PBA to bind and stabilise the smart materials. When glucose concentration increases, it competitively binds to PBA and destabilises the structure. Insulin will then be released into the environment (Figure 2.28).⁶² On the other hand, PBA can also use to bind gluconic acid modified insulin (G-insulin). Shiino and co-workers have used PBA containing gel beads to secure G-insulin within the beads.⁶³ Upon competitive binding from free glucose, G-insulin will be released.

Figure 2.28. Nanoparticles with glucose molecules crosslinked with PBA.⁶³ Glucose competitively binds to PBA on the polymer, resulting in dissociation of the nanoparticle assemblies and release of insulin.

PBA has been used to chemically modify insulin for glucose responsive release systems (Figure 2.29A).^{64, 65} Hoeg-Jensen and co-workers have created a modified insulin by adding a glucose-like diol and PBA to LysB29 residue (Figure 2.29B).⁶⁵ Without glucose, PBA of one insulin molecule binds to the diol moiety of another insulin molecule and forms aggregates. As glucose concentration increases, it competitively binds to PBA and insulin aggregates dissociate into individual molecules.

Figure 2.29. Chemically modified insulin with addition of a glucose-like diol and PBA molecule.⁶⁵ (A) Chemical structure of the modified insulin. (B) Illustration of aggregation between the chemically modified insulins and its dissociation in the presence of glucose.

2.3 Overview of cancer and targeted drug delivery

Cancer is a group of diseases characterised by uncontrollable growth and spreading of malignant cells. It is one of the leading causes of death in the world, with nearly 22 million people expected to suffer from cancer yearly by 2030. The occurrence of cancer is affected by many factors such as genetics, diet and gender. While the exact cancer treatment options depend on the type and stage of cancer, most cancer can be treated by surgery, chemotherapy or radiotherapy. While surgeries are often used to remove tumours, it cannot prevent metastasis of the malignant cells. Radiotherapy uses radiation to shrink the size of tumour and kill cancerous cells. However, the effectiveness of radiation is limited by lifetime dose limit and potential damage to neighbouring health tissues. Chemotherapy uses cytotoxic drugs mainly through intravenous administration for inducing cancer cell death, shrinking tumours or reducing symptoms of cancer.

Nonetheless, these cytotoxic drugs are usually non-selective and can lead to undesirable side effects such as haemorrhaging or anaemia. Therefore, alternative approaches have been considered using targeted drug delivery to improve effectiveness and reduce side effects of chemotherapy.

2.3.1 Biomedical applications of Magnetic Nanoparticles (MNPs)

MNPs are particles with magnetic properties in nanoscale dimensions (usually between 10 to 100 nm), which can be manipulated by application of an external magnetic field.⁶⁶ Biomedical applications of MNPs can be classified into either analytical or therapeutic applications (Figure 2.30).⁶⁷ In analytical applications, MNPs have been commonly used as biosensors for sensing inter-molecules binding events, magnetic carriers in purification experiments, and contrast agents for MRI scanning.⁶⁷⁻⁶⁹ In therapeutic applications, MNPs are used as carriers for delivering different drugs or biomolecules into cells along with MHT in cancer therapy.^{67, 70-73} The small size range of MNPs has the advantage of enabling them to penetrate both the blood capillaries and cells. Of the different MNPs available, iron oxide-based superparamagnetic MNPs are the most preferred for therapeutic applications due to their low toxicity.^{74, 75} In cancer therapy, the use of MNPs in targeted delivery and MHT are the most popular applications.⁷⁶⁻⁸⁴

Figure 2.30. Biomedical applications of MNPs. (A) Analytical applications. (B) Therapeutic applications.⁶⁷

2.3.2 Targeted delivery using MNPs

The direct delivery of chemotherapeutic drugs is often inefficient and maybe degraded during the process. Also, chemotherapeutic drugs such as doxorubicin (Dox) are generally non-specific and often needs to be administrated intravenously.⁸⁵ Systemic delivery exposes healthy tissues to these chemotherapeutic drugs, resulting in many side effects. Hence, developing a targeted drug delivery system is one way to overcome this non-specificity. Targeted drug delivery is defined as drug accumulation at a diseased site, independent of the route and method of administration.^{86, 87} This is different from targeted therapy where there is a specific binding between drug and its receptor. Four crucial factors (retain, evade, target and release) affects the efficiency of targeted drug delivery⁸⁸ The delivery vehicle must retain in the circulation long enough to reach its target site while evading rapid clearance from the liver and kidneys. Next, the delivery must accumulate at its target site and release its contents upon reaching its destination. Incorporating MNPs into the drug delivery systems or using MNPs as drug carriers are popular methods for targeted drug delivery.

2.3.3 Targeted delivery by co-encapsulation

One way of using magnetic field for targeted delivery is by encapsulating the drug together with MNPs, making magnetic micro- or nano-capsules. Sabnis and co-workers have created reconstituted high-density lipoprotein (rHDL) nanoparticles, loaded with valrubicin, a cancer drug and superparamagnetic iron oxide nanoparticles (SPION) (Figure 2.31A).⁸⁹ These nanoparticles were concentrated in cells nearer to the magnetic field, but not in cells that were far from the magnetic field (Figure 2.31B).⁸⁹ This shows that it is possible to control the movement and concentrate these magnetic capsules in areas of interest using magnetic field.

Beside HDL nanoparticles, MNPs and chemotherapeutic drugs such as Dox can also be co-encapsulate into suitable polymer nanoparticles. Yanhui *et al* co-encapsulated both

MNPs and Dox into a poly(lactic-co-glycolic acid) (PLGA) based polymeric nanocarrier using a single emulsion evaporation technique (Figure 2.32A).⁹⁰ The encapsulation efficiency of Dox in these nanoparticles was close to 90%.⁹⁰ The nanoparticles exhibited a pH responsive behaviour, where the amount of Dox released for 7 days at pH 5 was 40 % more than at pH 7.4 (Figure 2.32B). These MNPs+Dox nanoparticles were uptake into Lewis lung carcinoma (LLC) cells efficiently, triggering apoptosis and/or necrosis in a dose-dependent manner.⁹⁰ Also, MNPs+Dox nanoparticles (80%) were more efficient in inducing LLC cell death when compared to MNPs alone (20%) or free Dox alone (29%) treatments (Figure 2.32C). Similarly, results from *in vivo* cancer mouse model demonstrated that only MNPs+Dox nanoparticles together with an external magnetic field could shrink the volume of tumour (Figure 2.32D).⁹⁰

Figure 2.31. Reconstituted high-density lipoprotein (rHDL) nanoparticles for magnetic targeted delivery.⁸⁹ (A) Illustration of rHDL-valrubicin-SPION nanoparticles. (B) Prostate cancer cells were treated with rHDL-valrubicin-SPION nanoparticles, followed by Prussian blue staining to reveal MNPs. Left: Cells are subjected to directional magnetic field, before staining; Middle: cells that are near to magnetic field; Right: cells that are farther from magnetic field.

Figure 2.32. Characterisation of MNP+Dox loaded PLGA nanoparticles.⁹⁰ (A) TEM micrograph. (B) *In vitro* Dox release profiles at pH 5 and pH 7.4. ($n = 3$, average) (C) Percentages of live, apoptotic and necrotic cells when treated with MNPs only, free Dox only and Dox+MNPs PLGA nanoparticles. (D) Mean LLC tumour volume after different treatments. ($n = 6$ to 10, mean $\pm$ S.D.)

2.3.4 Targeted delivery by Drug-conjugated MNPs

The other method of targeted delivery is by loading drugs directly onto MNPs or to the surface coatings of these MNPs.^{91-94 90} However, drug-conjugated MNPs may suffer from low drug loading due to limitation capacity of the thin coating on top of MNPs. Also, MNPs may suffer from aggregation, changing its size and drug release kinetics. To overcome these problems, surface functionalization of MNPs can be done to prevent agglomeration and non-specific interactions.⁶⁶ Song and co-workers have increased the drug loading capacity of SPION by coating it with two different layers of polymers.⁹⁵ The SPION was first coated with a single silica coating, followed by a mPEG-PLA layer (Figure 2.33A). The SiO₂ protects the SPION while increasing the surface area for subsequent polymer coating. The double coated SPION (Fe₃O₄-SiO₂-Polym (I) and (II))

had much higher loading capacity (~190X) of paclitaxel compared to a single coated SPION (Fe_3O_4 -Polym).⁹⁵ Although both Polym (I) and (II) are identical in its composition and coating, Polym(II) had higher loading capacity compared to polym(I) due to its thicker coating (Figure 2.33B).⁹⁵

Figure 2.33. Dual coating of SPION for greater drug loading capacity.⁹⁵ (A) SPION was first coated with SiO_2 , then a subsequent second layer of mPEG-PLA polymer was coated onto Fe_3O_4 - SiO_2 to form the final Fe_3O_4 - SiO_2 -Polym. (B) Dual coated SPION had higher drug loading compared to single coated SPION. The loading capacity increases with the thickness of second coating. ($n = 3$)

2.3.5 Magnetic hyperthermia

In addition to magnetic targeting, MNPs can be used to generate heat under AMF, commonly known as MHT. It can act as a kind of cancer therapy that utilise heat to induce stress or apoptosis within the tumour cells.⁹⁶⁻⁹⁸ Besides inducing cell death, hyperthermia is commonly used as an adjunctive treatment together with radiotherapy and chemotherapy to enhance their effectiveness.⁹⁹ Depending on the temperature, MHT is classified as thermoablation ($>46^\circ\text{C}$), moderate hyperthermia (42°C to 46°C) and diathermia ($<42^\circ\text{C}$). Only thermoablation and moderate hyperthermia are being used in cancer treatments, whereas diathermia is used for rheumatic diseases.⁹⁹ Besides acting directly on tumour

cells, MHT can also be used for triggering release of drugs encapsulated in heat-sensitive polymers.

Zhang and co-workers have proposed using a combination of moderate hyperthermia and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) for treating glioblastoma (Figure 2.37A).¹⁰⁰ A nanocomplex solution consisting of TRAIL, SPIONs and poly(organophosphazene) (PPZ) polymer were prepared prior to gelation. Upon reaching 37 °C, gelation would occur, to entrap the TRAIL and SPIONs within the hydrogel (T/S-NHs). MHT was then used to trigger the release of TRAIL into the surrounding by inducing T/S-NHs dissolution at higher temperatures. *In vivo* results shown that tumour volume decreased significantly when subjected to both T/S-NHs and hyperthermia treatment (Figure 2.37B).¹⁰⁰ Histology of treated tumours were stained with terminal deoxynucleotidyl transferase dUTP Nick-End Labelling (TUNEL) assay to reveal the apoptosis cells. The resulting green fluorescence were observed to be stronger in tumour treated with combination of T/S-NHs and hyperthermia than those with T/S-NHs treatment only (Figure 2.37C).¹⁰⁰

Besides hydrogels mention above, MNPs-based polymer micelles (MNPs-PMs) have also been developed for thermo-chemotherapy. In this drug delivery system, MNPs was used to trigger drug release and for MHT while PMs act as a Dox reservoir.⁸¹ The PMs were made from poly(ethylene glycol)-poly(lactide) (PEG-PLA) because of its ability to self-assembly at physiological temperature, reduced protein adsorption, and decreased non-specific uptake.⁸¹ PEG-PLA was conjugated to citric acid-coated MNPs through an amidation reaction. MNPs-PMs were able to reach a temperature of 45 °C (temperature needed for therapeutic hyperthermia) with 25 min of AMF treatment (Figure 2.38A). Dox released from Dox-loaded MNPs-PMs was much faster in the presence of AMF or at 45 °C.⁸¹ In the absence of AMF and at room temperature, only ~ 20% of Dox was release from Dox-loaded MNPs-PMs over a period of 24 hours, suggesting that Dox was trapped within the hydrophobic PLA (Figure 2.38B). Cell viability of Dox-loaded MNPs-PMs treated A549 lung cancer cells decreased significantly when compared to Dox only or

MNPs-PMs only treated cells, indicating that combined therapy was more effective than single treatments (Figure 2.38C).⁸¹

Figure 2.37. Combination therapy using moderate hyperthermia and TRAIL-loaded hydrogel.¹⁰⁰ (A) Illustration of gelation and dissolution of hydrogels at different temperature. Nanocomplex solution undergo gelation upon reaching 37 °C, trapping TRAIL and SPION within the hydrogel structure. MHT increased the temperature within the hydrogel, inducing dissolution and release of TRAIL. (B) Tumour volume after different T/S-NHs and hyperthermia treatments. Black arrows represent the day of MHT. The symbol ** and *** indicates the statistical significance at levels of $P < 0.01$ and of $P < 0.001$ respectively. ($n = 4$) (C) TUNEL staining of tumour tissues treated with T/S-NHs +/- MHT. Green fluorescence indicates cells that were undergoing apoptosis.

Figure 2.38. Characterisation of Dox-loaded MNPs-PMs.⁸¹ (A) Temperature measurements of MNPs-PM under AMF, with water acting as control. (B) Rate of Dox release from MNPs-PMs without AMF, with AMF and at 45 °C. (C) Cell viability of A549 cells treated with MNPs-PMs, free Dox or Dox-loaded MNPs-PMs in the presence or absence of AMF. ($n=3$, biological repeats, mean $\pm$ S.D.) A p -value of < 0.05 was considered to be statistically significant.

2.4 Research questions based on Literature review

2.4.1 Glucose responsive delivery system

There are 3 main methods of glucose sensing that have already been incorporated into drug delivery system namely, boronic acids, GOx and glucose binding proteins (GBPs). The binding of boronic acids to cis1,2- or 1,3-diols are reversible and strong, making them suitable to be used in biologically relevant situations. However, boronic acids does not bind to glucose only, making boronic acid-based insulin delivery system less accurate than other “smart” insulin delivery system. In fact, monoboronic acids such as PBA are known to have a higher selectivity for fructose than other monosaccharides.¹⁰¹ Overall, the specificity and affinity of monoboronic acid-based glucose responsive delivery systems are poor.¹⁰² To improve its binding selectivity, carefully positioning of multi-boronic acids is needed. While glucose-specific boronic acids have already been developed^{103, 104}, they are mainly integrated into glucose sensors and not into insulin delivery systems. The interaction between boronic acid and monosaccharides is also highly dependent on pH, making boronic acid-based delivery systems inaccurate in patients with extreme acidosis, a common diabetic complication.

GBP is a soluble periplasmic binding protein expressed in many bacteria, which changes its conformational upon glucose binding.¹⁰⁵ It has high selectivity for glucose but can also binds to galactose at a lower affinity.¹⁰⁶ Also, GBPs are not a type of enzymes, therefore they can be fully functional without any additional molecules such as co-factors.¹⁰⁶ The dissociation constant of wild-type GBPs is 0.2 μ M, making it saturated at blood glucose conditions (1.7 to 33 mM) normally monitored for diabetes.^{107, 108} Hence, the binding affinity of wild-type GBPs has to be significantly decreased before it can be used as a glucose sensor for diabetic patients.¹⁰⁹ Incorporation of wild-type GBPs into commercial devices has been impeded due to this limitation. To overcome this limitation, research has been done change the binding affinity by altering GBP amino sequence through mutagenesis and DNA shuffling.¹¹⁰⁻¹¹² Yet, the selectivity of these altered GBPs towards glucose and other sugars are still poorly understood¹⁰⁵ GBPs are also highly immunogenic, making it difficult to be used in drug delivery systems.¹¹³

GOx is the most common enzyme used in glucose biosensors due to its high selectivity for glucose.¹¹⁴ It is cheap, can be easily purified and is an extremely robust enzyme, able to endure greater extreme of ionic strength, temperature and pH when compared to other enzymes. Since the conversion of glucose to gluconic acid by GOx requires oxygen molecules, oxygen is a limiting factor for this reaction. Also, H₂O₂ that is produced during the reaction inhibits GOx, resulting in decreased enzyme activity.¹¹⁵ To overcome these limitations, a peroxidase such as catalase is often added to break down H₂O₂ and produce oxygen molecules at the same time.^{44, 116-119} The incorporation of multiple components (GOx, peroxidase and insulin) into a single delivery system is tedious and may eventually affect the encapsulation efficiency of individual components. Hence, aqueous-based delivery vehicles are preferred for optimal GOx activity by allowing rapid diffusion of oxygen and H₂O₂.

2.4.2 MNPs and Thermo-chemotherapy

Iron-based MNPs are biocompatible and their surfaces can be easily modified for loading chemotherapeutic drugs. However, MNPs are often rapidly cleared by the reticuloendothelial system (RES) or macrophages because of their size.¹²⁰ Once in the circulation, MNPs with hydrophobic surface are coated quickly by plasma proteins and are removed rapidly by immune system.¹²¹ On the other hand, MNPs with hydrophilic surface can stay in the circulation for longer time but may not be suitable for loading of hydrophobic drugs such as paclitaxel. MNPs tends to agglomerate even with surface coating due to its dipole-dipole interaction. In the absence of surface coating, MNPs are easily oxidised, resulting in loss of magnetisation.^{121, 122} Hence MNPs are often coated with a polymer such as PEG or co-encapsulated with a chemotherapeutic drug within delivery vehicle e.g. liposomes.

Thermo-chemotherapy has been found to be more effective than other chemotherapy only or hyperthermia only treatment. Therefore, the use of thermo-responsive polymers in drug delivery systems has gained interest in recent years. Indeed, most of the stimuli-responsive polymers that have been studied are thermo-responsive polymers. However, biodegradability and biocompatibility of most synthetic polymers

were in question and yet only few natural thermo-responsive polymers have been reported.^{123, 124} Poly(*N*-isopropylacrylamide) (PNIPAAm) is the most commonly used synthetic thermo-responsive polymer for its low toxicity and biocompatibility.¹²⁴⁻¹²⁶ However, its NIPAAm monomer is toxic and residual monomers may be present in PNIPAAm-based delivery vehicle such as hydrogels.¹²⁷ Long-term toxicity on these synthetic polymers also have not been studied. Tropoelastin or elastin-like polypeptides are a well-known natural thermo-responsive polymers^{128, 129} but are unsuitable for thermo-chemotherapy due to their lower-critical solution temperature, where heating drives self-assembly as opposed to dis-assembly that is needed for release of drug. Therefore, a new thermo-responsive natural material is desirable for fabrication of thermo-responsive drug delivery systems.

2.5 PhD in context of literature

Among the beak proteins of *D. gigas* squid beak, only DgHBPs have shown to undergo coacervation. The ability of DgHBPs to undergo coacervation is due to its tandem repeats located within the C-terminal region. Since encapsulation is the most important application of coacervation, this project will focus on the use of DgHBP-based coacervation to fabricate stimuli-responsive drug delivery systems, followed by characterisation of these delivery systems.

The first part of this project was to understand the coacervation conditions of DgHBPs and DgHBP derived peptides. The best-suited protein/peptide was then be used for subsequent studies. Stabilisation of the coacervates by different crosslinking methods would be explored next. Finally, characterisation and cytotoxicity of these crosslinked coacervates would also be studied. After an in-depth examination of coacervation properties, the author implemented these coacervates as drug delivery vehicles for different diseases. The second part of project involved the use of coacervates to create a GRIDS. Since coacervates are known to encapsulate large and small molecular weight macromolecules and to have a high loading capacity, the coacervation-based GRIDS were fabricated by co-encapsulation of GOx and insulin. The encapsulation efficiency, secondary structure, and *in vitro* release of insulin would also be evaluated. The last part

of project addressed the use of coacervates in thermo-chemotherapy. Since MNPs can be used for targeting and MHT, both MNPs and a chemotherapeutic drug, Dox was encapsulated within the coacervates. The efficacy of these coacervates as a thermo-responsive delivery vehicle for thermo-chemotherapy was examined extensively. The understanding and application of these coacervates into different delivery systems provide further information on the use of these biopolymers as a novel encapsulation material.

Reference

1. P. R. Boyle, *Cephalopod Life Cycles: Comparative reviews*, Academic Press, 1987.
2. C. Nigmatullin, *Fisheries Research*, 2001, **54**, 9-19.
3. U. M. W. F. Gilly, C. H. Baxter, B. A. Block, A. Boustany, L. Zeidberg, K. Reisenbichler, B. Robison, G. Bazzino and C. Salinas, *Marine Ecology Progress Series*, 2006, **324**, 1-17.
4. U. Markaida and O. Sosa-Nishizaki, *Journal of the Marine Biological Association of the United Kingdom*, 2003, **83**, 507-522.
5. S. H. Hiew and A. Miserez, *ACS Biomaterials Science & Engineering*, 2016, **3**, 680-693.
6. A. Miserez, J. C. Weaver, P. B. Pedersen, T. Schneeberk, R. T. Hanlon, D. Kisailus and H. Birkedal, *Advanced Materials*, 2009, **21**, 401-406.
7. D. Ding, P. A. Guerette, S. Hoon, K. W. Kong, T. Cornvik, M. Nilsson, A. Kumar, J. Lescar and A. Miserez, *Biomacromolecules*, 2014, **15**, 3278-3289.
8. J. Gatesy, C. Hayashi, D. Motriuk, J. Woods and R. Lewis, *Science*, 2001, **291**, 2603-2605.
9. P. A. Guerette, D. G. Ginzinger, B. H. Weber and J. M. Gosline, *Science*, 1996, **272**, 112-115.
10. R. V. Lewis, *Chem Rev*, 2006, **106**, 3762-3774.
11. P. A. Guerette, S. Hoon, D. Ding, S. Amini, A. Masic, V. Ravi, B. Venkatesh, J. C. Weaver and A. Miserez, *ACS Nano*, 2014, **8**, 7170-7179.
12. A. Miserez, Y. Li, J. H. Waite and F. Zok, *Acta Biomater*, 2007, **3**, 139-149.

13. A. Miserez, T. Schneberk, C. Sun, F. W. Zok and J. H. Waite, *Science*, 2008, **319**, 1816-1819.
14. Y. Tan, S. Hoon, P. A. Guerette, W. Wei, A. Ghadban, C. Hao, A. Miserez and J. H. Waite, *Nat Chem Biol*, 2015, **11**, 488-495.
15. E. Khor, *Chitin 2nd edition*, Elsevier, 2014.
16. D. J. Rubin, A. Miserez and J. H. Waite, in *Advances in Insect Physiology*, eds. J. Casas and S. J. Simpson, Academic Press, 2010, vol. 38, pp. 75-133.
17. L. E. Arnow, *Journal of Biological Chemistry*, 1937, **118**, 531-537.
18. A. Miserez, D. Rubin and J. H. Waite, *J Biol Chem*, 2010, **285**, 38115-38124.
19. D. R. Talham, *Crystal Growth & Design*, 2002, **2**, 675-675.
20. L. Wang and G. H. Nancollas, *Metal ions in life sciences*, 2010, **4**, 413-456.
21. L. Addadi, D. Joester, F. Nudelman and S. Weiner, *Chemistry*, 2006, **12**, 980-987.
22. B. R. Lawn, *Journal of the American Ceramic Society*, 2005, **81**, 1977-1994.
23. T. Nishino, R. Matsui and K. Nakamae, *Journal of Polymer Science Part B: Polymer Physics*, 1999, **37**, 1191-1196.
24. T. Wu, S. Zivanovic, F. A. Draughon, W. S. Conway and C. E. Sams, *J Agric Food Chem*, 2005, **53**, 3888-3894.
25. J. F. V. Vincent and S. Ablett, *Journal of Insect Physiology*, 1987, **33**, 973-979.
26. C. Giulivi, N. J. Traaseth and K. J. Davies, *Amino Acids*, 2003, **25**, 227-232.
27. O. Zvarec, S. Purushotham, A. Masic, R. V. Ramanujan and A. Miserez, *Langmuir*, 2013, **29**, 10899-10906.
28. J. D. Fox, P. D. Capadona Jr Fau - Marasco, S. J. Marasco Pd Fau - Rowan and S. J. Rowan.
29. D. L. Crimmins, S. M. Mische and N. D. Denslow, *Curr Protoc Protein Sci*, 2005, **Chapter 11**, Unit 11 14.
30. *The Protein Protocols Handbook*, Humana Press, 2002.
31. D. Sahal, R. Kannan, A. Sinha, V. Babbarwal, B. Gnana Prakash, G. Singh and V. S. Chauhan, *Anal Biochem*, 2002, **308**, 405-408.
32. D. L. Crimmins, S. M. Mische and N. D. Denslow, *Curr Protoc Protein Sci*, 2005, **Chapter 11**, Unit 11 14.
33. M. A. Frohman, G. R. Dush Mk Fau - Martin and G. R. Martin.

34. E. de Castro, C. J. Sigrist, A. Gattiker, V. Bulliard, P. S. Langendijk-Genevaux, E. Gasteiger, A. Bairoch and N. Hulo, *Nucleic Acids Res*, 2006, **34**, W362-365.
35. C. Hao, Ph.D, Nanyang Technological University, 2017.
36. H. Cai, B. Gabryelczyk, M. S. S. Manimekalai, G. Gruber, S. Salentinig and A. Miserez, *Soft Matter*, 2017, **13**, 7740-7752.
37. V. Tamburro Am Fau - Guantieri, D. Guantieri V Fau - Daga-Gordini, G. Daga-Gordini D Fau - Abatangelo and G. Abatangelo, *J Biol Chem*, 1978, **253**.
38. A. M. Brown and N. J. Zondlo.
39. B. Bochicchio, A. Ait-Ali, A. M. Tamburro and A. J. Alix, *Biopolymers*, 2004, **73**, 484-493.
40. R. G. Urry Dw Fau - Shaw, K. U. Shaw Rg Fau - Prasad and K. U. Prasad, *Biochem Biophys Res Commun*, 1985, **130**(1).
41. T. L. Urry Dw Fau - Trapane, K. U. Trapane Tl Fau - Prasad and K. U. Prasad, *Biopolymers*, 1985, **12**.
42. S. A. Jensen, B. Vrhovski and A. S. Weiss, *J Biol Chem*, 2000, **275**, 28449-28454.
43. A. M. Tamburro, V. Guantieri, D. Daga-Gordini and G. Abatangelo, *Biochimica et Biophysica Acta (BBA) - Protein Structure*, 1977, **492**, 370-376.
44. L. Zhao, L. Wang, Y. Zhang, S. Xiao, F. Bi, J. Zhao, G. Gai and J. Ding, *Polymers (Basel)*, 2017, **9**, 255.
45. M. J. Webber and D. G. Anderson, *J Drug Target*, 2015, **23**, 651-655.
46. H. Thabit and R. Hovorka, *Endocrinol Metab Clin North Am*, 2012, **41**, 105-117.
47. V. Dadlani, J. E. Pinsker, E. Dassau and Y. C. Kudva, *Curr Diab Rep*, 2018, **18**, 88.
48. D. Elleri, D. B. Dunger and R. Hovorka, *BMC Med*, 2011, **9**, 120.
49. W. Wu and S. Zhou, *Macromol Biosci*, 2013, **13**, 1464-1477.
50. S. Anastassiadis and I. G. Morgunov.
51. Y. H. Cui, P. Wei, F. Peng, M. H. Zong and W. Y. Lou, *RSC Advances*, 2018, **8**, 9970-9978.
52. S. B. Bankar, M. V. Bule, R. S. Singhal and L. Ananthanarayan, *Biotechnol Adv*, 2009, **27**, 489-501.

53. Z. Gu, T. T. Dang, M. Ma, B. C. Tang, H. Cheng, S. Jiang, Y. Dong, Y. Zhang and D. G. Anderson, *ACS Nano*, 2013, **7**, 6758-6766.
54. L. Y. Chu, Y. Li, J. H. Zhu, H. D. Wang and Y. J. Liang, *J Control Release*, 2004, **97**, 43-53.
55. W. Tai, R. Mo, J. Di, V. Subramanian, X. Gu, J. B. Buse and Z. Gu, *Biomacromolecules*, 2014, **15**, 3495-3502.
56. B. Pappin, M. J and T. A, 2012, DOI: 10.5772/50630.
57. A. Resendez, M. A. Halim, J. Singh, D. L. Webb and B. Singaram, *Org Biomol Chem*, 2017, **15**, 9727-9733.
58. G. H.S, R. M. Melavanki, N. D, B. P and R. A. Kusanur, *Journal of Molecular Liquids*, 2017, **227**, 37-43.
59. X. Sun, W. Zhai, J. S. Fossey and T. D. James, *Chem Commun (Camb)*, 2016, **52**, 3456-3469.
60. V. Ravaine, C. Ancla and B. Catargi, *J Control Release*, 2008, **132**, 2-11.
61. X. Sun and T. D. James, *Chem Rev*, 2015, **115**, 8001-8037.
62. O. Veis, B. C. Tang, K. A. Whitehead, D. G. Anderson and R. Langer, *Nat Rev Drug Discov*, 2015, **14**, 45-57.
63. D. Shiino, Y. Murata, A. Kubo, Y. J. Kim, K. Kataoka, Y. Koyama, A. Kikuchi, M. Yokoyama, Y. Sakurai and T. Okano, *Journal of Controlled Release*, 1995, **37**, 269-276.
64. D. H. Chou, M. J. Webber, B. C. Tang, A. B. Lin, L. S. Thapa, D. Deng, J. V. Truong, A. B. Cortinas, R. Langer and D. G. Anderson, *Proc Natl Acad Sci U S A*, 2015, **112**, 2401-2406.
65. T. Hoeg-Jensen, S. Havelund, P. K. Nielsen and J. Markussen, *J Am Chem Soc*, 2005, **127**, 6158-6159.
66. A. K. Mitra, V. Agrahari, A. Mandal, K. Cholkar, C. Natarajan, S. Shah, M. Joseph, H. M. Trinh, R. Vaishya, X. Yang, Y. Hao, V. Khurana and D. Pal, *J Control Release*, 2015, **219**, 248-268.
67. P. Tartaj, M. P. Morales, T. Gonzalez-Carreño, S. Veintemillas-Verdaguer, O. Bomati-Miguel, A. G. Roca, R. Costo and C. J. Serna, in *Reference Module in*

- Materials Science and Materials Engineering*, Elsevier, 2016, DOI: <https://doi.org/10.1016/B978-0-12-803581-8.02251-7>.
68. M. Faraji, *Nanochemistry Research*, 2016, **1**, 264-290.
69. J. S. Beveridge, J. R. Stephens and M. E. Williams, *Annu Rev Anal Chem (Palo Alto Calif)*, 2011, **4**, 251-273.
70. A. Akbarzadeh, M. Samiei and S. Davaran, *Nanoscale Res Lett*, 2012, **7**, 144.
71. Y. Bao, T. Wen, A. C. Samia, A. Khandhar and K. M. Krishnan, *J Mater Sci*, 2016, **51**, 513-553.
72. R. A. Frimpong and J. Z. Hilt, *Nanomedicine (Lond)*, 2010, **5**, 1401-1414.
73. Q. A. Pankhurst, J. Connolly, S. K. Jones and J. Dobson, *Journal of Physics D: Applied Physics*, 2003, **36**, R167-R181.
74. H. Markides, M. Rotherham and A. J. El Haj, *Journal of Nanomaterials*, 2012, **2012**, 1-11.
75. T. K. Jain, M. K. Reddy, M. A. Morales, D. L. Leslie-Pelecky and V. Labhasetwar, *Mol Pharm*, 2008, **5**, 316-327.
76. M. Li, W. Bu, J. Ren, J. Li, L. Deng, M. Gao, X. Gao and P. Wang, *Theranostics*, 2018, **8**, 693-709.
77. P. M. Price, W. E. Mahmoud, A. A. Al-Ghamdi and L. M. Bronstein, *Front Chem*, 2018, **6**, 619.
78. G. K. Thirunavukkarasu, K. Cherukula, H. Lee, Y. Y. Jeong, I. K. Park and J. Y. Lee, *Biomaterials*, 2018, **180**, 240-252.
79. N. Ž. Knežević, I. Gadjanski and J.-O. Durand, *Journal of Materials Chemistry B*, 2019, **7**, 9-23.
80. A. Hervault and N. T. Thanh, *Nanoscale*, 2014, **6**, 11553-11573.
81. H. C. Kim, E. Kim, S. W. Jeong, T. L. Ha, S. I. Park, S. G. Lee, S. J. Lee and S. W. Lee, *Nanoscale*, 2015, **7**, 16470-16480.
82. S. Y. Wang, M. C. Liu and K. A. Kang, *Adv Exp Med Biol*, 2013, **765**, 315-321.
83. O. L. Gobbo, K. Sjaastad, M. W. Radomski, Y. Volkov and A. Prina-Mello, *Theranostics*, 2015, **5**, 1249-1263.
84. J. Owen, Q. Pankhurst and E. Stride, *Int J Hyperthermia*, 2012, **28**, 362-373.

85. C. A. Papadimitriou, M. A. Dimopoulos, C. Ampela, A. Louvrou-Fertaki, A. Anagnostopoulos, P. Athanassiades, S. Stamatelopoulos and A. Keramopoulos, *Oncology*, 1998, **55**, 533-537.
86. P. Utreja, S. Jain and A. K. Tiwary, *Curr Drug Deliv*, 2010, **7**, 152-161.
87. D. Rosenblum, N. Joshi, W. Tao, J. M. Karp and D. Peer, *Nat Commun*, 2018, **9**, 1410.
88. J. Chomoucka, J. Drbohlavova, D. Huska, V. Adam, R. Kizek and J. Hubalek, *Pharmacol Res*, 2010, **62**, 144-149.
89. S. Sabnis, N. A. Sabnis, S. Raut and A. G. Lacko, *Int J Nanomedicine*, 2017, **12**, 1453-1464.
90. Y. Jia, M. Yuan, H. Yuan, X. Huang, X. Sui, X. Cui, F. Tang, J. Peng, J. Chen, S. Lu, W. Xu, L. Zhang and Q. Guo, *Int J Nanomedicine*, 2012, **7**, 1697-1708.
91. J. Wong, J. Prout and A. Seifalian, *Curr Pharm Des*, 2017, **23**, 2908-2917.
92. Z. Q. Samra, M. Ahmad S Fau - Javeid, N. Javeid M Fau - Dar, M. S. Dar N Fau - Aslam, I. Aslam Ms Fau - Gull, M. M. Gull I Fau - Ahmad and M. M. Ahmad.
93. S. Nigam, K. C. Barick and D. Bahadur, *Journal of Magnetism and Magnetic Materials*, 2011, **323**, 237-243.
94. O. Aras, G. Pearce, A. J. Watkins, F. Nurili, E. I. Medine, O. K. Guldu, V. Tekin, J. Wong, X. Ma, R. Ting, P. Unak and O. Akin, *PLoS One*, 2018, **13**, e0202482.
95. L. Song, M. Sun, Y. Zhao and Z. Wang, *RSC Advances*, 2014, **4**, 4893.
96. B. T. Mai, P. B. Balakrishnan, M. J. Barthel, F. Piccardi, D. Niculaes, F. Marinaro, S. Fernandes, A. Curcio, H. Kakwere, G. Autret, R. Cingolani, F. Gazeau and T. Pellegrino, *ACS Appl Mater Interfaces*, 2019, **11**, 5727-5739.
97. D. Chang, M. Lim, J. Goos, R. Qiao, Y. Y. Ng, F. M. Mansfeld, M. Jackson, T. P. Davis and M. Kavallaris, *Front Pharmacol*, 2018, **9**, 831.
98. P. Das, M. Colombo and D. Prosperi, *Colloids Surf B Biointerfaces*, 2019, **174**, 42-55.
99. V. M. Salunkhe Ab Fau - Khot, S. H. Khot Vm Fau - Pawar and S. H. Pawar.
100. Z. Q. Zhang and S. C. Song, *Biomaterials*, 2017, **132**, 16-27.
101. J. P. Lorand and J. O. Edwards, *The Journal of Organic Chemistry*, 1959, **24**, 769-774.

102. X. Wu, Z. Li, X. X. Chen, J. S. Fossey, T. D. James and Y. B. Jiang, *Chem Soc Rev*, 2013, **42**, 8032-8048.
103. T. D. James, K. R. A. S. Sandanayake and S. Shinkai, *Angewandte Chemie International Edition in English*, 1994, **33**, 2207-2209.
104. W. Zhai, L. Male and J. S. Fossey, *Chem Commun (Camb)*, 2017, **53**, 2218-2221.
105. S. Joel, K. B. Turner and S. Daunert, *ACS Chem Biol*, 2014, **9**, 1595-1602.
106. L. Tolosa and G. Rao, in *Glucose Sensing*, eds. C. D. Geddes and J. R. Lakowicz, Springer US, Boston, MA, 2006, DOI: 10.1007/0-387-33015-1_13, pp. 323-331.
107. L. L. Salins, R. A. Ware, C. M. Ensor and S. Daunert, *Anal Biochem*, 2001, **294**, 19-26.
108. S. Joel, S. Turner Kb Fau - Daunert and S. Daunert.
109. T. J. Amiss, D. B. Sherman, C. M. Nycz, S. A. Andaluz and J. B. Pitner, *Protein science : a publication of the Protein Society*, 2007, **16**, 2350-2359.
110. C. Jackel, P. Kast and D. Hilvert, *Annu Rev Biophys*, 2008, **37**, 153-173.
111. T. J. Amiss, D. B. Sherman, C. M. Nycz, S. A. Andaluz and J. B. Pitner, *Protein Sci*, 2007, **16**, 2350-2359.
112. A. Sakaguchi-Mikami, A. Taneoka, R. Yamoto, S. Ferri and K. Sode, *Biotechnol Lett*, 2008, **30**, 1453-1460.
113. G. Tiegs, J. Hentschel and A. Wendel, *J Clin Invest*, 1992, **90**, 196-203.
114. E. H. Yoo and S. Y. Lee, *Sensors (Basel)*, 2010, **10**, 4558-4576.
115. J. Bao, K. Furumoto, M. Yoshimoto, K. Fukunaga and K. Nakao, *Biochemical Engineering Journal*, 2003, **13**, 69-72.
116. M. J. Webber and D. G. Anderson, *Journal of drug targeting*, 2015, **23**, 651-655.
117. M. Y. Kim and J. Kim, *ACS Biomaterials Science & Engineering*, 2017, **3**, 572-578.
118. Z. Gu, T. Dang, M. Ma, B. Tang, H. Cheng, S. Jiang, Y. Dong, Y. Zhang and D. G. Anderson, *ACS nano*, 2013, **7**.
119. L. A. Klumb and T. A. Horbett, *Journal of Controlled Release*, 1992, **18**, 59-80.
120. E. B. Denkbaş, E. Çelik, E. Erdal, D. Kavaz, Ö. Akbal, G. Kara and C. Bayram, in *Nanobiomaterials in Drug Delivery*, ed. A. M. Grumezescu, William Andrew

- Publishing, 2016, DOI: <https://doi.org/10.1016/B978-0-323-42866-8.00009-5>, pp. 285-331.
121. U. Gaur, T. K. Sahoo Sk Fau - De, P. C. De Tk Fau - Ghosh, A. Ghosh Pc Fau - Maitra, P. K. Maitra A Fau - Ghosh and P. K. Ghosh.
 122. M. Mikhaylova, D. K. Kim, N. Bobrysheva, M. Osmolowsky, V. Semenov, T. Tsakalakos and M. Muhammed, *Langmuir*, 2004, **20**, 2472-2477.
 123. W. B. Liechty, D. R. Kryscio, B. V. Slaughter and N. A. Peppas, *Annu Rev Chem Biomol Eng*, 2010, **1**, 149-173.
 124. Y.-J. Kim and Y. T. Matsunaga, *Journal of Materials Chemistry B*, 2017, **5**, 4307-4321.
 125. H. Malonne, F. Eeckman, D. Fontaine, A. Otto, L. D. Vos, A. Moes, J. Fontaine and K. Amighi, *Eur J Pharm Biopharm*, 2005, **61**, 188-194.
 126. L. H. Lima, Y. Morales and T. Cabral, *J Ophthalmol*, 2016, **2016**, 5356371.
 127. M. A. Cooperstein and H. E. Canavan, *Biointerphases*, 2013, **8**, 19.
 128. B. Vrhovski, S. Jensen and A. S. Weiss, *Eur J Biochem*, 1997, **250**, 92-98.
 129. G. C. Yeo, F. W. Keeley and A. S. Weiss, *Adv Colloid Interface Sci*, 2011, **167**, 94-103.

Chapter 3

Experimental Methodology

This chapter describes the materials and experimental procedures used in this project and is divided into 3 sections. The first section presents the methodology used in characterisation and crosslinking of coacervates, followed by use of DgHBP-2 peptide coacervates in the preparation of a glucose responsive insulin delivery system in the second section. In vitro release assays for studying the release profiles of insulin are also described in the second section. The third section summarises the techniques used in the fabrication of Dox-loaded magnetic coacervates (DMCs) for targeted delivery and thermo-chemotherapy. Cell uptake of coacervates and cytotoxicity of DMCs are also elaborated in the third section.

3.1 Characterisation and crosslinking of coacervates

3.1.1 Relative turbidity for coacervation studies

In order to determine the optimum conditions for coacervation, the turbidity of the coacervates formed in different buffer conditions was measured to infer the level of coacervation.¹ This method is known as turbidimetry and measures the intensity decrease of the transmitted light after being scattered by the particles within the solution.^{2, 3} These particles are the coacervates within its respective buffer solution. The degree of turbidity by these solutions is often measured using an ultraviolet-visible spectrophotometer. Here, coacervation for different buffer conditions was measured at wavelength of 600 nm using UV-vis spectrophotometry (Lambda 35, Perkin Elmer). Turbidity measurement was done with the following procedures:

1. Coacervation of proteins/peptide was induced by adding them to respective buffers.
2. Incubation of coacervates at room temperature for 30s to allow completion of coacervation.
3. Dispersion of coacervates by pipetting.
4. Pipette the coacervates into a plastic cuvette (10 mm pathlength) for turbidity measurement.

Each measurement was done in triplicate and relative turbidity is then calculated by³:

$$100 - 100 * [10^{-A_{600}}]$$

Where A_{600} is the absorbance of the coacervates at 600 nm.

Since this method measures only the degree of light scattering, any aggregates within the solution can contribute to the level of turbidity. Hence, optical microscopy of the coacervates was done to determine if aggregates were present.

3.1.2 Optical and fluorescence microscopy

Optical microscopy is one of the earliest microscopes developed in the 17th century.⁴ It uses a group of optical lenses coupled with visible light to increase magnification of the images from a sample. This set of optical lenses enlarged the sample into a virtual image which is subsequently captured by a light sensitive camera.^{4, 5} Modern cameras such as charged-coupled device cameras allow the conversion of light information to digital images and live-viewing of image directly on a computer screen.

Fluorescence microscope is very similar to optical microscope except that it uses a higher intensity light source such as xenon light lamp to illuminate the sample.⁶ The sample needs to contain a fluorescence group to be used in fluorescence microscopy. The strong light source excites the fluorescence group, which subsequently emits light of longer wavelength.⁶ This light is then used to construct the magnified image.

Here, the coacervates were first added to a glass coverslip and then incubated for 5 min at room temperature. This incubation was to allow the coacervates to attach onto the surface of coverslip for easy visualisation. The coacervates were then imaged on an inverted microscope (Axio observer z1, Zeiss) in transmission mode, equipped with a 63X oil Immersion lens. Micrographs were imaged and processed with Zen imaging (Blue edition, Zeiss) and Photoshop (Adobe) software.

3.1.3 Dynamic light scattering (DLS)

Macromolecules undergo random motion in solution, a phenomenon called Brownian motion.^{7, 8} When light is scattered from these moving macromolecules, this result in a random change (destructive or constructive interference) to the scattered light phase. These fluctuations in the intensity of scattered light is then used to measure and correlate to the hydrodynamic diameters of macromolecules.⁸ The average size of macromolecules can then be calculated and are frequently presented as a distribution profile, which displays the intensity of detected particles.^{7, 8}

In this project, DLS measurements of coacervates were done on a ZetaPAL particle size analyzer (Brookhaven Instruments Corporation, USA) fitted with a 35mW red diode laser (640 nm, 90° scattering angle). DgHBP-2 peptide coacervates were first pipetted 10 times to ensure homogeneity. Next, the dispersed coacervates were pipetted into a plastic cuvette (Eppendorf, Germany) for DLS measurement. The measurements were done at 25°C and at a detection angle of 90°. The results were reported by averaging 5 different measurements and plotted with originPro 8.6 software.

3.1.4 Solid phase peptide synthesis (SPPS) of DgHBP-2-peptide DOPA

SPPS is a famous and frequently used peptide synthesis technique for short peptides, invented by Bruce Merrifield in 1963.⁹ Since its discovery, various improvement and automation such as microwave synthesis and automated peptide synthesizers, have been done to enhance its efficiency and peptide length.^{10, 11} SPPS method comprises of repetitive coupling and deprotection process, where amino acids (with N-terminus protection) e.g. 9-Fluorenylmethyloxycarbonyl (Fmoc) and tert-butyloxycarbonyl (Boc) protected amino acids are attached to a resin solid support in a step by step manner, starting from the C-terminus.^{9, 12} Of the two common N-terminus protected amino acids, Fmoc is more preferred due to its easier synthesis and purification process, and also fewer toxic reagents needed for peptide cleavage from the resins after peptide synthesis.¹⁰

A typical SPPS protocol consisted of activation, deprotection, coupling and cleavage steps (Figure 3.1).⁹ Chlorotriyl chloride resins were used for the synthesis of pepHBP-1 and DgHBP-2 peptide DOPA in this project.

Figure 3.1. General peptide synthesis by SPPS method. C-terminus of the last amino acid is coupled to the solid support resin through a linker. The peptide is then elongated by cycles of i) Deprotection of N-terminus group (NPG); ii) activation of carboxy group, and iii) coupling of next amino acid to the previous one. The final polypeptide and SPG are then cleaved off from the resin. Aa and SPG represent amino acid and side-chain protecting group.

In general, chlorotriyl chloride resin was first swelled in anhydrous Dimethylformamide (DMF) within the reaction vessel. Activation+coupling cocktail containing Fmoc-AA-OH, N,N-Diisopropylcarbodiimide (DIC) and 4-Dimethylaminopyridine (DMAP) in anhydrous Dichloromethane (DCM) were subsequently added to the swelled resins. The activation + coupling cocktail and resin mixture was then stirred together and incubated overnight. The unreacted amino acids were washed off and capping of the unreacted resin was done using DCM, methanol and DIPEA mixture. After capping, the beads were washed and subsequent deprotection and coupling of next amino acid was done.

2,4,6-trinitrobenzene sulfonic acid (TNBS) test is used to test for the presence of primary amino groups and is done after each coupling and deprotection steps.¹¹ This is to evaluate the level of completeness in coupling and deprotection reactions. The presence of primary amino groups in the resins will turn the resins orange whereas the absence of primary amino groups in resins remain colourless. Hence, a successful deprotection and complete coupling will result in orange and colourless resins respectively. Upon completion of the synthesis, the final peptide and its SPG was cleaved off the resin using an acid such as Trifluoroacetic acid (95%) and a suitable scavenger chemical such as Triisopropylsilyl (2.5%). The peptide was immediately washed and purified using reverse phase HPLC.

The yield of peptide can reach up to grams scale, depending mainly on the type of solid support used, peptide length and peptide sequence. The likelihood of the peptide to aggregate during synthesis increases with the hydrophobicity of the peptide, resulting in decrease overall yield. Truncation of peptides also becomes obvious as the peptide length increases, decreasing its yield with increasing length of peptide. Despite these limitations, SPPS is still a popular method to reach large scale peptide synthesis.

3.1.5 Reverse-phase High performance liquid chromatography (RP-HPLC)

HPLC is a kind of liquid chromatography that is commonly used for separating and purifying molecules. The pump forces the sample together with a solvent (mobile phase)

through a column (contains stationary phase) at high pressure for analysing and separation of a sample mixture.¹³ The columns are packed with different resins that are made of different material, different pore sizes and surface functionalisation.¹⁴ Two common columns used in protein purification are for size exclusion (SEC) and reverse phase chromatography (RPC). SEC separate protein molecules based on molecular size by eluting larger molecules first. RPC separate protein molecules based on their polarity where hydrophilic molecules elute faster than hydrophobic molecules.¹³

In RP-HPLC, the mobile phase is usually hydrophilic whereas the stationary phase is hydrophobic.¹³ Therefore, hydrophobic molecules within the sample will bind to the stationary phase and elute later compared to hydrophilic molecules. Purification of DgHBP-2 peptide DOPA was done with a C18 reverse phase preparative column (Kromasil, Sweden) and ACN as mobile phase. After purification, trace analysis of DgHBP-2 peptide DOPA was done on a C18 analytical column (Agilent technologies, USA) to determine its purity.

3.1.6 Matrix assisted laser desorption or ionisation- time of flight mass spectrometry (MALDI-TOF)

MALDI-TOF is a method where samples are first ionised into charged molecules and measures the ratio of molecular weight to charge of the sample.¹⁵ Samples are dispersed within a matrix, which are involved in the ionisation of the sample. The matrix used is dependent on the type of sample.^{15, 16} Sinapinic acid was used as matrix in this study. The sample-matrix mixture is pipetted and dried on a stainless-steel metal plate. After inserting the metal plate into the MALDI-TOF instrument, a laser ionised the sample and the sample molecules become charged (Figure 3.2).¹⁵⁻¹⁷ These charged molecules are accelerated through an electric field and move towards the detector. Linear and reflector mode are the common detection modes used by the detector.¹⁵ In linear detection mode, the charged molecules move on a linear flight path, where their mass/charge (m/z) ratio is calculated by their time required to reach the detector. In reflector detection mode, the charged molecules are reflected by an ion mirror to reach its designated detector. Reflector mode is more accurate while the linear mode is more sensitive.

Figure 3.2. Ionisation of sample molecules in Maldi-TOF.¹⁵

Here, sample preparation was done using the sandwich method.¹⁸ Sinapinic acid was first added and dried on the MALDI plate. The sample was then added on top of the dried sinapinic acid. After the sample had dried, sinapinic acid was added on top of the sample. This mixture was air dried before inserting into Shimadzu Biotech TOF machine for analysis.

3.1.7 Arnow staining

Arnow staining is specific for catechol containing samples such as DOPA. A colour change from colourless to red will be seen when tested samples contain catechol groups.^{19,}
²⁰ This colour change is due to nitration of catechol that eventually led to production of a red colour diphenolate derivative.²⁰

The sample was first acidified using 0.5M HCL. Nitrile-molybdate reagent (1:1 sodium nitrite and sodium molybdate) was immediately added to the acidified sample. Sodium hydroxide was subsequently added to neutralise the reaction mixture. These reagents were added in the specified order and thorough mixing was done after each addition. The absorbance of the reaction mixture was then measured using a UV/VIS spectrophotometer. Tyrosine and L-3,4-dihydroxyphenylalanine were used as a negative and positive control respectively.

3.1.8 Crosslinking of DgHBP-2 peptide DOPA coacervates

L-3,4-dihydroxyphenylalanine (DOPA) is an unnatural amino acid that is derived from tyrosine, with an additional hydroxyl group on its aromatic ring, leading to a catechol group. Catechol groups are known to be reactive, can interact with both inorganic and organic compounds. There are two main catechol crosslinking method: coordination and covalent bond (Figure 3.3).²¹ Transition metals such as Fe can form coordination bonds with catechols to become catechol-metal complexes. Coordination bond is a kind of chemical bond where one atom contributes both electrons of the covalent bond.^{22, 23} These metal coordination complexes are found to be highly tuneable and reversible.²¹ Catechols can also undergo oxidation easily to form o-quinones. Catechol oxidation can be achieved through exposure to air, enzymatic or chemical oxidation.²¹ O-quinones are extremely reactive electrophilic molecules and can undergo chemical reactions with amines/thiols to form covalent crosslinking.^{21, 23} Crosslinking is used to increase the stability of coacervates and allow free drugs to be wash away after encapsulation step.

Figure 3.3. Coordination and covalent crosslinking of catechols.²¹

In this project, DgHBP-2 peptide DOPA coacervates were first diluted to 500 μL in their respective buffer, followed by the addition of a transition metal salt (e.g. FeCl_3) or chemical oxidant (e.g. sodium periodate, NaIO_4) to the diluted coacervates to a final

concentration of 0.4 mM and 2.4 mM, respectively. The final mixture was subsequently vortexed at low speed for 60s. After crosslinking, the crosslinked coacervates were washed 3X with deionised water.

3.1.9 Crosslinking of DgHBP-2 peptide coacervates

4-methylcatechol and sodium periodate

DgHBP-2 peptide sequence has the natural amino acid tyrosine instead of DOPA. In order to incorporate catechols for crosslinking, 4-methylcatechol (4-MC) was added. Similarly to DOPA, 4-MC has a catechol group and can be chemically oxidised NaIO_4 . By controlling the amount of 4-MC added, the level of crosslinking can be controlled to a greater extent.

In summary, 4-MC was first added to DgHBP-2 peptide coacervates followed by dilution with phosphate buffer to a final volume of 500 μL . A chemical oxidant such as NaIO_4 was next added and vortexed to mix the reaction evenly. After crosslinking, the crosslinked coacervates were washed with deionised water to remove excess 4-MC and NaIO_4 .

Dityrosine crosslinking

Since the sequence of DgHBP-2 peptide contains a high percentage of tyrosine (~20%), DgHBP-2 peptide coacervates can be crosslinked by inducing dityrosine formation. Dityrosine is formed when tyrosine residues are subjected to oxidative stress. Dityrosine bonds are found throughout nature and has been used to stabilise naturally occurring structural materials such as resilin and silk proteins.²⁴ Also, they are easily formed in physiological conditions, making them useful in biomedical applications. The formation of dityrosine crosslinking can be facilitate by enzymes, transition metals and photoinitiated catalysts.²⁴ The first step of dityrosine formation is the formation of tyrosyl radical, through the removal of hydrogen atom from the hydroxyl group on the phenol group of tyrosine. The tyrosyl radical then reacts with another nearby tyrosyl radical to form dityrosine. Trityrosine can also occurs when a tyrosyl radical first reacts with a tyrosine, allowing its unpaired electron to react on the third tyrosyl radical.²⁴

Here, riboflavin was chosen as a photoinitiators for dityrosine crosslinking due to its low toxicity.^{25, 26} During the crosslinking process, riboflavin enhanced the production of radicals by ultraviolet A light (UVA), oxidising tyrosine residues to tyrosyl radical. Two main types of reactions have been proposed. In type 1 reaction, the energy from UVA is absorbed by riboflavin leading to generation of triplet-excited riboflavin. In type II reactions, triplet oxygen ($^3\text{O}_2$) is produced through the interaction of singlet oxygen ($^1\text{O}_2$), the former oxygen species then oxidise the tyrosine residues (Figure 3.4). The formation of dityrosine in coacervates was analysed by fluorescence because of its autofluorescence properties.²⁴

Figure 3.4. Mechanisms of dityrosine crosslinking by riboflavin and UVA.²⁴ (UVA: ultraviolet light A; ISC: intersystem crosslinking and RF: riboflavin)

3.1.10 Field Emission Scanning Electron Microscopy (FESEM)

Scanning electron microscope take images using a concentrated, high energy electron beam generated by a field emitter gun such as lanthanum hexaboride electron gun.²⁷ Condenser and objective lens within the SEM are used to focus the beam onto the

sample.²⁸ Once the electrons hit and penetrate the sample, back-scattered and secondary electrons are produced depending on the sample density and accelerating voltage of the beam.²⁷ In addition to backscattered and secondary electrons, x-rays that are characteristics of the sample are also produced. Emitted electrons are collected by their corresponding detectors and converted into digital images.

FESEM was used to study the surface morphology of crosslinked coacervates. Samples were air-dried overnight under ambient conditions on carbon tape and then coated with platinum to decrease surface charging. Platinum coating was done at these conditions: Current: 20mA, Pressure: 5 Pa and Duration: 15s. SEM Micrographs were taken using Field Emission Scanning Electron Microscope (7600F, JSM Schottky).

3.1.11 Transmission Electron Microscopy (TEM)

TEM is based on similar principles as light microscope except that it uses electrons instead of visible light.²⁹ Since the wavelength of electrons is smaller than light, the resolution of TEM is much higher than light microscopy. In TEM, a beam of electrons is focused onto the sample by a series of condenser lenses.²⁹ The number of electrons transmitted through the sample is dependent on the thickness and electron transparency of the sample.³⁰ Objective lenses are often used to improve image contrast by blocking out high angle diffracted electrons. These transmitted electrons are then collected on a charge coupled device camera and processed into digital images.³⁰ Dark areas within an image signify lesser electrons have transmitted through due to denser or thicker regions. In contrast, bright areas within the image signify more electrons have transmitted through and that region is less dense or thinner. TEM was used to study the density of coacervates and confirmed the presence of MNPs within the coacervates.

In this project, crosslinked coacervates were added onto a copper TEM grid (01824, Ted Pella) and air-dried overnight under ambient conditions. Micrographs were taken by a Transmission Electron Microscope (Libra Plus, Carl Zeiss).

3.1.12 Zeta potential

Zeta potential is the potential difference between the still layer of dispersion medium on top of the particle and its surrounding mobile dispersion medium.³¹ The value of zeta potential is important due to its correlation with stability of colloid solutions.³² Particles with low zeta potential tends to aggregate or flocculate while particles with high zeta potential can resist flocculation and remains stable.³³ When the zeta potential is high, attractive forces are lower than repulsive forces, resulting in a stable colloid system.

To measure the zeta potential of particles, an electric field is applied onto the solution.³⁴ Particles will subsequently move towards the direction opposite to their surface charge, at a speed that is relative to their electrical charge. When the solution is illuminated with a light source such as laser, the motion of the particles scattered the light, creating a Doppler shift that corresponds to the speed of particle. Hence, zeta potential is determined by calculating the change in frequency of scattered light.³²

Zeta potential of coacervates were measured in a zeta potential plastic cell using a nano particle analyser (Sz-100, Horiba). Each sample was measured 3 times and the data was averaged to get the final zeta potential.

3.1.13 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay

MTT assay measures the reduction of MTT reagent by NAD(P)H-dependent cellular oxidoreductase enzymes to infer cellular metabolic levels as cell viability.^{35, 36} After MTT is added to cells, it enters the mitochondria and is reduced into an insoluble dark purple formazan product. This purple formazan product is solubilised by an organic solvent and then measured on a spectrophotometer. Higher absorbance indicates higher amount of metabolically active cells.

In summary, cells were seeded into wells of a 96-well plate (Thermo Fisher Scientific). 100 μ L of media containing different concentrations of coacervates were added to the cells and incubated for another 24 hours. 10 μ L of MTT reagent (stock solution: 5 mg/mL) was next added per well. After 4 hours of incubation at 37°C, excess MTT reagent

and media was removed by vacuum aspiration. 100 μ L of DMSO was then pipetted into each well to dissolve the formazan crystals. The absorbance of the dissolved crystals was measured immediately on a microplate reader (Infinity M200, Tecan) at 570 nm. Native DMSO was used as a blank. Relative cell viability is calculated using the below equation:

$$(A_t - A_b)/(A_c - A_b) * 100$$

where A_t , A_b , and A_c designate average absorbance value of wells containing tested cells, native DMSO and control cells correspondingly.

3.1.14 Live/dead staining

Live/dead staining is a popular cell viability assay that are based on the concurrent staining of live and dead cells with two different fluorescence probes.³⁷ These two probes are calcein AM and ethidium homodimer-1 (EthD-1), which measures intracellular esterase activity and plasma membrane integrity respectively.³⁷ Enzymes with esterase activity are ubiquitously present inside viable cells. Calcein AM is a cell permeable and non-fluorescent by itself. Once Calcein AM enter a viable cell, enzymes will hydrolase it to calcein which is both highly fluorescence and cell impermeable.³⁷ Calcein get trapped inside live cells and the cells eventually emits uniform green fluorescence. EthD-1 is a cell impermeable probe that cannot enter live cells. When cells undergoing cell death, cell membrane will lose its integrity. EthD-1 will then be able to enter the cell and binds to nucleic acids.³⁷ Upon binding to nucleic acid, the fluorescence of EthD-1 will increase 40X.

MTT assay infer the viability of cells by testing on the metabolic rate of cells whereas live/dead staining directly assess the physical and biochemical activity of the cells. By using both methods to test for cell viability, the accuracy of the results will be increased. Cells were seeded onto 24-well culture plates or 35mm dishes (Corning Incorporated) and incubated at 37°C overnight. Culture media containing coacervates were then added to the cells. After another 24 hours of incubation at 37°C, cells were washed with PBS and subjected to live/dead staining (LIVE/DEAD[®] Viability/Cytotoxicity kit, Invitrogen).

After 20 min of incubation at room temperature, imaging of the cells was done by an inverted microscope (Axio observer z1, Zeiss).

3.2 Glucose responsive insulin delivery system

3.2.1 Labelling of Insulin and GOx

Amine reactive fluorescence probes have been commonly used to modify biomolecules such as proteins and peptides.³⁸ They are often used to prepare bioconjugates for downstream experiments such as immunochemistry and cell tracking. There are 3 main types of amine reactive dyes: succinimidyl esters, isothiocyanates and sulfonyl chlorides.³⁹ Among the three types, isothiocyanates is the most widely used fluorescence probe. Isothiocyanate moiety is produced from isocyanate group by replacing the latter's oxygen with a sulphur. The isothiocyanate group reacts with amines, resulting in the formation of a stable thioureal linkage between the fluorescence probe and biomolecule (Figure 3.5).³⁹

Since isothiocyanates are amine-reactive, buffers such as Tris and glycine buffers that contain free amines are not suitable to be used during the reaction process. Ammonium salts should also be removed from the sample before labelling to prevent the formation of unstable intermediates.³⁸ Also, the reaction is strongly dependent on pH and may not be suitable for all biomolecules labelling. Despite these limitations, fluorescence isothiocyanate probes remains the most popular fluorescent labelling dyes. Insulin and GOx were labelling in this project for easy visualisation under fluorescent microscope.

Figure 3.5. Chemical reaction of isothiocyanate with biomolecules to form thiourea.

Fluorescein isothiocyanate (FITC) or Rhodamine B isothiocyanate (RITC) was first dissolved in acetone, then added dropwise into respective buffers containing either GOx or insulin. The mixture was then incubated in the dark with constant stirring for 16 hours at

room temperature. Excess unreacted dye was removed by a gel filtration column (PD-10, GE healthcare life sciences) and eluted with phosphate buffer.

3.2.2 Encapsulation of Drug by coacervation

Coacervates have been shown to sequester macromolecules, additional of peptide into the drug-containing buffer will induce encapsulation and coacervation simultaneously.^{40,41} Drugs were first added to respective buffer to their final concentrations. Peptide was next added to the drug-containing buffer to trigger encapsulation and coacervation. Pipetting was promptly done and then incubated for 5 min at room temperature to allow complete encapsulation/coacervation.

3.2.3 Fluorescence emission assay for insulin-FITC and Dox

Fluorescence spectroscopy measures the light emitted from a sample when it is excited by an external light source such as ultraviolet light. Molecules within the sample absorb the light energy (of certain wavelength) and assume an excited electronic singlet state. When the molecules go back to its original ground state, a photon of light energy (of longer wavelength) is emitted as fluorescence.⁴²

Insulin-FITC and Dox fluorescence spectra were measured using a spectrofluorometer (Fluorolog, Horiba). Insulin-FITC was excited at 490 nm and its fluorescence spectra were measured from 500 nm to 550 nm. Dox was excited at 488 nm and its emission was measured from 500 nm to 600 nm. A quartz cuvette was used for measuring the fluorescence.

3.2.4 Encapsulation efficiency (EE%) (Fluorescence method)

EE% is the percentage of drug that has been encapsulated successfully into coacervates. Drug-loaded coacervates were removed by centrifugation at a speed of 13000 rpm for 5 min. The supernatant was then collected for fluorescence measurement (Fluorolog, HORIBA Jobin Yvon). The concentration of drug within the supernatant was back-calculated using a standard curve. The EE% is calculated with the equation below:

$$EE\% = \frac{\text{Total Drug} - \text{Free Drug}}{\text{Total Drug}} \times 100\%$$

where Total Drug represents the initial Drug concentration and Free Drug represents the concentration of unencapsulated Drug measured within supernatant.

3.2.5 Encapsulation efficiency (EE%) (RP-HPLC method)

To confirm the EE% results from fluorescence assay, RP-HPLC is used to separate and quantify the amount of native insulin from DgHBP-2 peptide within the supernatant after encapsulation. By comparing the insulin peak from the chromatograph before and after encapsulation, the amount of insulin that has been encapsulated can be known.

Insulin-loaded coacervates were first centrifuged and the supernatant was diluted to 5% ACN. The pellet was similarly solubilised using 5% ACN. Both supernatant and pellet were then analysed using RP-HPLC fitted with an analytical column (C18, Agilent).

3.2.6 *In vitro* insulin release assay

In vitro release assay is a measurement of rate of release of drug from a drug-product matrix under a pre-determined laboratory setting.⁴³ This assay involves exposing the dosage form to conditions that induce release of drug and measures the amount of drug release over a period of time under these conditions.⁴³ These conditions are chosen to simulate *in vivo* conditions. It is a crucial assay to determine differences between prototypes, provide insight on *in vivo* behaviour and estimating timeframe of drug release.^{43, 44} Glucose was used as a trigger to induce insulin release from the coacervates-based GRIDS. 1 mg/mL was used to simulate fasting glucose level while 4 mg/mL was used to simulate hyperglycaemia.

Glucose responsive time point assay

Insulin-FITC and/or GOx-loaded DgHBP-2 peptide coacervates were first diluted with phosphate buffer. The diluted coacervates were pipetted into dialysis tubes and subsequently dialysed against phosphate buffer, with/without glucose for 48 hours at 37°C.

Fresh buffer was replaced at respective time points and the dialysed buffer was analysed by a fluorescence spectrophotometer (Fluorolog, HORIBA Jobin Yvon).

Alternating glucose concentration assay

Diluted insulin and/or GOx-loaded peptide coacervates were dialysed against phosphate buffer with 1 or 4 mg/mL of glucose for 9 hours at 37°C. Glucose concentration was interchanged between 1 and 4 mg/mL every 90 min. This alternating glucose concentrations were to mimic fluctuations in blood glucose level during fasting and after meal. The dialysed buffer analysed and then substituted at specific time points with fresh buffers.

3.2.7 Circular Dichroism (CD) Spectroscopy

CD is the difference between the absorption of left-handed and right-hand circularly polarised light.^{45, 46} This difference occurred when a molecule contains more than one light-absorbing groups (chiral chromophores). These non-zero CD signals can be either negative or positive, depending on which circularly polarised light is being absorbed to a greater extend. A CD spectrum is a compilation of CD signals across a range of wavelengths and can display as either negative or positive peaks.⁴⁶ CD spectroscopy are often used in the study of biological molecules like proteins or peptides to analyse its folding and secondary structure. When proteins or peptides adopt certain conformation e.g. α -helix, the chromophores of amides (from the backbone of polypeptide) become arranged in specific arrays and eventually give rise to its characteristic CD spectra [α -helix (222 nm, 208 nm negative bands and 193nm positive band); β -sheets (218 nm negative band and 195nm positive band) and disordered structure (195 nm negative band and weakly positive 210nm band)] (Figure 3.6).⁴⁶

Insulin molecule is comprised of two different peptide chains (chain A and B) linked by 3 disulfide bonds.⁴⁷ Insulin is consisted of mainly α -helixes with no beta strands and this secondary conformation is important for its biological function. To assess if encapsulation and its subsequent release from coacervates will alter the secondary structure of insulin, CD spectroscopy was used to compare native and released insulin.

In this work, native insulin, released insulin and DgHBP-2 peptide were diluted to their respective concentrations and their CD spectra were analysed with a quartz cuvette. Data collection was done on an AVIV CD spectrometer (Aviv Biomedical, USA). The final spectrum of each sample was an average of 3 different measurements.

Figure 3.6. Characteristic CD spectra of different secondary structures.⁴⁶

3.2.8 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA is a 96-well plate immunological assay frequently used to measure proteins/antibodies/antigen in biological samples.⁴⁸ The wells of these plates are coated with either antigens or antibodies. The type of ELISA used for detecting insulin was a sandwich ELISA (Figure 3.7). In this method, two types of antibodies are used to detect insulin within a sample. The first set of antibodies (capture antibody) are coated onto the 96 well plate and is used to capture insulin within a sample. Any unbound macromolecules within the sample will be washed off. The second set of antibodies (detection antibody) are labelled with enzyme such as alkaline phosphatase. Detection antibodies will then bind to the previously bounded insulin. After that, a substrate will be added and converted to a chromogenic product, which correlates to the concentration of insulin.⁴⁸

The binding between antibody and its respective epitope depends on the 3D conformation of the antigen. Hence, the binding of anti-insulin to released insulin suggests that its conformation remains unchanged. Since the function of insulin strongly depends on its native conformation, ELISA can be used to infer the biological activity of released insulin.

Figure 3.7. Principle of sandwich ELISA assay.⁴⁸ Monoclonal antibodies specific for the antigen of interest are coated onto the wells. After the sample is added to the wells, antigens within the sample will bound to the antibody. Enzyme-linked secondary antibodies are then added to the wells. These secondary antibodies will bind to the antigens and remained within the well after washing. Substrate is next added and converts to coloured product by antibody-bounded enzyme. (E: enzyme and S: substrate)

3.3 Dox-loaded magnetic coacervates

3.3.1 Magnetic alignment of coacervates

Without external magnetic field, the individual magnetic moments within MNPs are oriented randomly and their magnetic field cancel out each other.⁴⁹ When an external magnetic field is applied, the magnetic moments align themselves within the magnetic field. Hence, to determine if the magnetic coacervate were magnetically responsive, magnetic coacervates were subjected to an external magnetic field.

A uniform magnetic field was produced by 2 identical Nd-Fe-B permanent magnets that are opposite each other. A glass coverslip containing the magnetic coacervates was

then placed on top of these two magnets. After 5 min, the magnetic coacervates were observed and imaged by an inverted microscope (Axio observer z1, Zeiss).

3.3.2 Confocal microscopy

Confocal microscopy, commonly known as laser confocal scanning microscopy is like conventional microscopy except that it does not illuminate the whole sample at the same time. It uses light from a laser beam and focuses on a defined location of an explicit depth within the sample.⁵⁰ A spatial pinhole within the optical pathway is used to block off out of focus optical information, allowing only information from illuminated spot to reach the detector during image acquisition. In contrast, light information above and below the focal point contributes to the acquisition of conventional microscopy image.⁵¹ Therefore, confocal micrographs have better contrast and optical resolution compared to conventional micrographs.⁵⁰ Since out of focus light information is removed from the image plane, the resulting micrographs are sharper when compared to conventional microscope. One important disadvantage of confocal microscopy is the rapid photobleaching of sample due to the intense illumination of laser used in the microscopy. Approaches to reduce photobleaching include reducing excitation intensity, shorter scanning times and using high numerical aperture objectives.

During confocal microscopy, samples are scanned in a raster pattern to form an image of a single optical plane. Three dimensions of the sample can be formed by scanning sequential optical planes and merging them (z-stack) using a deconvolution software.^{50, 52} Besides z-stack images, vertical optical sectioning in y-z and x-y planes can be also performed using the confocal software. Here, confocal micrographs were taken with an inverted SP8 True Confocal Scanner microscope (Leica, Germany) and exported using Leica Application Suite X software (LAS X)

3.3.3 Endosomal staining with lysotracker

Endocytosis is a process where particles *e.g.* parts of cells and nutrients are taken into cells.⁵³ This process is used by all cells as most important molecules cannot diffuse freely through the plasma membrane. Endocytosis is needed for various cell functions that

are critical for cellular homeostasis.⁵⁴ During this internalisation process, cell membrane invagination occurs to form a membrane-bound compartment called endosomes. There are two classical types of endocytosis, phagocytosis and pinocytosis, which are the cellular uptake of large particles and fluid respectively (Figure 3.8).⁵⁴ Pinocytosis has been further divided into clathrin-mediated and clathrin-independent endocytosis (CME and CIE, respectively).

Figure 3.8. Different classification of endocytosis in mammalian cells.⁵⁵ CME and CIE represent clathrin-mediated endocytosis and clathrin-independent endocytosis, respectively.

Since endocytosis is one of the main cell uptake process, endosomal staining was done to determine if coacervates are uptake into the cells and if they are inside endosomes after cellular uptake. Here, lysotracker probes were used to label endosomes in HepG2 cells. These probes are comprised of basic amines linked to a fluorophore, are partially protonated at physiological pH and are cell-permeable. Endosomes becomes acidic as they mature due to the presence of vacuolar membrane proton pumps (vacuolar-type H^+ -ATPase).⁵⁶ Lysotracker probes become fully protonated at acidic pH and get selectively accumulate within acidic endosomes.

To better study the mechanism of endocytosis, cell biologists have been using chemical or genetic means to inhibit endocytosis.⁵⁵ These inhibitors help to reveal the physiological consequences of inhibiting the molecules needed for specific endocytosis pathways.⁵⁵ While genetic approaches to inhibit endocytosis are more specific,

overexpression of proteins that are involved in endocytosis can lead to many indirect effects on different pathways. Depleting proteins by silencing are timing consuming, allowing cells to adapt to the loss of protein.⁵⁵ Therefore, chemical inhibitors are used in this study because they can be added easily to inhibit any endocytosis pathway of interest. By evaluating the cellular uptake of coacervates in cells that have been treated with these inhibitors, the mode of cellular uptake for coacervates can be assessed. In these experiments, cells were first treated with respective endocytosis inhibitors for 1 hour, followed by additional of green fluorescence protein (GFP)-loaded coacervates. After 4 hours of incubation, the cells were washed and stained with Hoechst 33342 and wheat germ agglutinin-Alexa Fluor 633. The coacervates-treated cells were then assessed by confocal microscopy.

3.3.4 Heating mechanism of MNPs under AMF

The two main mechanisms of MHT in magnetic materials are hysteresis and relaxation losses. The dominant mechanism for heat production is dependent on the size of magnetic materials involved. Larger MNPs contain multiple magnetic domains which randomly orientated. In the presence of AMF, these magnetic domains continuously align themselves in the direction of the magnetic field.⁵⁷ This continuous change in alignment results in a difference in energy that is released as heat (Figure 3.9). When the MNPs are small enough (≤ 128 nm), each MNPs only has a single magnetic domain and are deemed superparamagnetic. In the presence of AMF, heating in superparamagnetic MNPs is mainly due to Neel and Brownian relaxation (Figure 3.9).⁵⁷ Neel relaxation occurs when the magnetic domain undergoes rapid changes in AMF and this rearrangement is opposed by the crystalline structure of MNPs, resulting in heat production.⁵⁸ Brownian relaxation occurs from the physical rotation of MNPs within a medium in AMF, resulting in frictional heat generation. This physical rotation occurs when the MNPs are trying to align themselves within the AMF.⁵⁸

Figure 3.9. Mechanisms of heat generation (MHT) by MNPs in AMF.⁵⁷

References

1. D.M.Lawler, *Journal*, 2016, 152-163.
2. T. Kourti, *IEEE Control Systems*, 2002, **22**, 10-25.
3. W. Wei, L. Petrone, Y. Tan, H. Cai, J. N. Israelachvili, A. Miserez and J. H. Waite, *Adv Funct Mater*, 2016, **26**, 3496-3507.
4. A. Di Gianfrancesco, in *Materials for Ultra-Supercritical and Advanced Ultra-Supercritical Power Plants*, ed. A. Di Gianfrancesco, Woodhead Publishing, 2017, DOI: <https://doi.org/10.1016/B978-0-08-100552-1.00008-7>, pp. 197-245.
5. J. Bergström, in *Mechanics of Solid Polymers*, ed. J. Bergström, William Andrew Publishing, 2015, DOI: <https://doi.org/10.1016/B978-0-323-31150-2.00002-9>, pp. 19-114.
6. F. Rost, in *Encyclopedia of Spectroscopy and Spectrometry*, ed. J. C. Lindon, Elsevier, Oxford, 1999, DOI: <https://doi.org/10.1006/rwsp.2000.0090>, pp. 565-570.
7. J. Stetefeld, S. A. McKenna and T. R. Patel, *Biophys Rev*, 2016, **8**, 409-427.
8. B. Lorber, F. Fischer, M. Bailly, H. Roy and D. Kern, *Biochem Mol Biol Educ*, 2012, **40**, 372-382.
9. G. B. Fields, *Curr Protoc Protein Sci*, 2002, **Chapter 18**, Unit 18 11.
10. R. Behrendt, P. White and J. Offer, *J Pept Sci*, 2016, **22**, 4-27.
11. I. Coin, M. Beyermann and M. Bienert, *Nat Protoc*, 2007, **2**, 3247-3256.

12. J. M. Palomo, *RSC Adv.*, 2014, **4**, 32658-32672.
13. M. V. Moreno-Arribas and M. C. Polo, in *Encyclopedia of Food Sciences and Nutrition (Second Edition)*, ed. B. Caballero, Academic Press, Oxford, 2003, DOI: <https://doi.org/10.1016/B0-12-227055-X/00232-7>, pp. 1274-1280.
14. S. D. America, Introduction to HPLC, <https://www.shodexhplc.com/lessons/lesson-1-introduction-to-hplc/>, (accessed 20-05-2019, 2019).
15. MALDI-TOF Mass Spectrometry, <https://www.creative-proteomics.com/technology/maldi-tof-mass-spectrometry.htm>, (accessed 27-06-2019, 2019).
16. N. Singhal, M. Kumar, P. K. Kanaujia and J. S. Virdi, *Front Microbiol*, 2015, **6**, 791.
17. tankeshwar, MALDI-TOF Mass Spectrometry: Principle and Applications in Microbiology, <https://microbeonline.com/maldi-tof-ms-principle-applications-microbiology/>).
18. MALDI Sample Preparation Sandwich Crystallization, <https://msr.dom.wustl.edu/maldi-sample-preparation-sandwich-crystallization/>, (accessed 25-05-2019, 2019).
19. O. Zvarec, S. Purushotham, A. Masic, R. V. Ramanujan and A. Miserez, *Langmuir*, 2013, **29**, 10899-10906.
20. R. E. Pankratz and F. J. Bandelin, *J Am Pharm Assoc Am Pharm Assoc*, 1956, **45**, 364-366.
21. J. Yang, M. A. Cohen Stuart and M. Kamperman, *Chem Soc Rev*, 2014, **43**, 8271-8298.
22. J. Yang, V. Saggiomo, A. H. Velders, M. A. Cohen Stuart and M. Kamperman, *PLoS One*, 2016, **11**, e0166490.
23. H. Xu, J. Nishida, W. Ma, H. Wu, M. Kobayashi, H. Otsuka and A. Takahara, *ACS Macro Letters*, 2012, **1**, 457-460.
24. B. P. Partlow, M. B. Applegate, F. G. Omenetto and D. L. Kaplan, *ACS Biomaterials Science & Engineering*, 2016, **2**, 2108-2121.
25. D. R. Cardoso, S. H. Libardi and L. H. Skibsted, *Food Funct*, 2012, **3**, 487-502.

26. M. B. Applegate, B. P. Partlow, J. Coburn, B. Marelli, C. Pirie, R. Pineda, D. L. Kaplan and F. G. Omenetto, *Adv Mater*, 2016, **28**, 2417-2420.
27. S. Swapp, Scanning Electron Microscopy (SEM), https://serc.carleton.edu/research_education/geochemsheets/techniques/SEM.html, (accessed 27-06-2019, 2019).
28. W. C. Nixon, *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 1971, **261**, 45-50.
29. C. Y. Tang and Z. Yang, in *Membrane Characterization*, eds. N. Hilal, A. F. Ismail, T. Matsuura and D. Oatley-Radcliffe, Elsevier, 2017, DOI: <https://doi.org/10.1016/B978-0-444-63776-5.00008-5>, pp. 145-159.
30. Transmission Electron Microscopy (TEM), <https://warwick.ac.uk/fac/sci/physics/current/postgraduate/regs/mpagswarwick/ex5/techniques/structural/tem/>, (accessed 20-05-2019, 2019).
31. G. W. Lu and P. Gao, in *Handbook of Non-Invasive Drug Delivery Systems*, ed. V. S. Kulkarni, William Andrew Publishing, Boston, 2010, DOI: <https://doi.org/10.1016/B978-0-8155-2025-2.10003-4>, pp. 59-94.
32. Z. Ding, Y. Jiang and X. Liu, in *Nanotechnology-Based Targeted Drug Delivery Systems for Brain Tumors*, eds. P. Kesharwani and U. Gupta, Academic Press, 2018, DOI: <https://doi.org/10.1016/B978-0-12-812218-1.00012-9>, pp. 327-358.
33. M. Gumustas, C. T. Sengel-Turk, A. Gumustas, S. A. Ozkan and B. Uslu, in *Multifunctional Systems for Combined Delivery, Biosensing and Diagnostics*, ed. A. M. Grumezescu, Elsevier, 2017, DOI: <https://doi.org/10.1016/B978-0-323-52725-5.00005-8>, pp. 67-108.
34. Principles of Zeta potential, <http://vlab.amrita.edu/?sub=3&brch=209&sim=600&cnt=1>, (accessed 25-05-2019, 2019).
35. *Bangladesh Journal of Pharmacology*, 8 April, 2017, 115-118.
36. P. Kumar, A. Nagarajan and P. D. Uchil, *Cold Spring Harb Protoc*, 2018, **2018**, pdb prot095505.
37. *Journal*, 21-12-2005.
38. D. Zicha, *Journal of Microscopy*, 2000, **198**, 159-159.

39. Introduction to Amine Modification, <https://www.thermofisher.com/sg/en/home/references/molecular-probes-the-handbook/fluorophores-and-their-amine-reactive-derivatives/introduction-to-amine-modification.html>, (accessed 15-05-2019, 2019).
40. D. Garenne, L. Beven, L. Navailles, F. Nallet, E. J. Dufourc and J. P. Douliez, *Angew Chem Int Ed Engl*, 2016, **55**, 13475-13479.
41. S. Deshpande, F. Brandenburg, A. Lau, M. G. F. Last, W. K. Spoelstra, L. Reese, S. Wunnava, M. Dogterom and C. Dekker, *Nat Commun*, 2019, **10**, 1800.
42. J. A. Holcombe, in *Encyclopedia of Spectroscopy and Spectrometry*, ed. J. C. Lindon, Elsevier, Oxford, 1999, DOI: <https://doi.org/10.1006/rwsp.2000.0009>, pp. 560-565.
43. Drug Release Testing, <https://www.particlesciences.com/services/drug-release-testing/>, (accessed 05-05-2019, 2019).
44. E. Nemutlu, İ. Eroğlu, H. Eroğlu and S. Kır, *Current Analytical Chemistry*, 2019, **15**, 373-409.
45. K. N. Nina Berova, Robert W. Woody, *Circular Dichroism: Principles and Applications, 2nd Edition*, April 2000.
46. N. J. Greenfield, *Nat Protoc*, 2006, **1**, 2876-2890.
47. Insulin Molecule, <https://www.worldofmolecules.com/life/insulin-molecule.html>).
48. P. Pokhrel, ELISA- Principle, Types and Applications, <http://microbiologynotes.com/elisa-principle-types-and-applications/>, (accessed 25-06-2019, 2019).
49. Alignment in Magnetic Fields, https://bio.groups.et.byu.net/mri_training_b_Alignment_in_Magnetic_Fields.phtml, (accessed 25th june 2019, 2019).
50. J. P. Robinson, in *Methods in Cell Biology*, Academic Press, 2001, vol. 63, pp. 89-106.
51. Confocal Microscopy, <https://ibidi.com/content/216-confocal-microscopy>).
52. Introductory Confocal Concepts, <https://www.microscopyu.com/techniques/confocal/introductory-confocal-concepts>, (accessed 19-05-2019, 2019).

53. S. L. Schmid, A. Sorkin and M. Zerial, *Cold Spring Harb Perspect Biol*, 2014, **6**, a022509.
54. C. G. Sunderland, *The Cell: A Molecular Approach*. 2nd edition.
55. D. Dutta and J. G. Donaldson, *Cell Logist*, 2012, **2**, 203-208.
56. M. E. Finbow and M. A. Harrison, *Biochem J*, 1997, **324 (Pt 3)**, 697-712.
57. D. Chang, M. Lim, J. Goos, R. Qiao, Y. Y. Ng, F. M. Mansfeld, M. Jackson, T. P. Davis and M. Kavallaris, *Front Pharmacol*, 2018, **9**, 831.
58. S. A. Shah, D. B. Reeves, R. M. Ferguson, J. B. Weaver and K. M. Krishnan, *Phys Rev B Condens Matter Mater Phys*, 2015, **92**, 094438.

Chapter 4

Characterisation and crosslinking of coacervates

Simple coacervation is a phenomenon where macromolecules phase separates into two different liquid phases: a concentrated coacervate phase and a diluted phase. Since encapsulation is the main application of coacervation and DgHBPs have shown to undergo simple coacervation, this chapter investigates the use of coacervates based from recombinant protein or biomimetic peptides as a material for 'smart' delivery systems. Of the different proteins and peptides tested, DgHBP-2 peptide derived from C-terminal of DgHBP-2 was found to be best suited material for encapsulation. Incorporation of unnatural amino acid, DOPA and use of chemical crosslinkers were also done to further stabilised the coacervates. Characterisation of these crosslinked coacervates and in vitro cytotoxicity assays were subsequently done to determine their suitability for fabrication of delivery systems.

Part of this section is published as Zhi Wei Lim, Yuan Ping, Ali Miserez, *Glucose-Responsive Peptide Coacervates with High Encapsulation Efficiency for Controlled Release of Insulin*, Bioconjugate Chemistry 2018 29 (7), 2176-2180

Part of this section is under revision as Zhi Wei Lim, Vijaykumar B. Varma, Raju V. Ramanujan, Ali Miserez, *Magnetically Responsive Peptide Coacervates with Dual Triggered Hyperthermia and Chemotherapy for Liver Cancer Treatment*, Biomaterial Science.

4.1 Introduction

In recent years, coacervates have becoming increasingly popular in the field of drug delivery, due to their ability to encapsulate different therapeutics or other molecules.¹⁻⁵ Encapsulation of therapeutics provide advantages over unencapsulated therapeutics such as protection against degradation, ease of administration and controlled release. Compared to other encapsulation methods, coacervates have the advantages of being able to undergo fast, self-assembly in an aqueous environment.¹ Therefore, bioactivity of protein/peptide drug are not affected as organic solvents are not used in the encapsulation or coacervation process.

The most well-known protein or peptide known to under simple coacervation in nature are tropoelastin or elastin-like polypeptides (ELP)^{6, 7} Since its discovery, many research has been done to understand the relationship between sequence and coacervation properties of tropoelastin and ELPS, as well as their potential applications. However, tropoelastin or ELP coacervation are through an inverse temperature transition, which may not be suitable for temperature triggered controlled release of therapeutics or thermo-chemotherapy. Recently, two families of squid beak proteins have been isolated and sequenced.⁸ Out of the two families of proteins, DgHBPs are of notably interest due to its ability to undergo simple coacervation. The coacervation properties were due to its tandem His- and Gly- rich repeats, located at the C-terminal.^{8, 9}

In this chapter, the optimum coacervation conditions of recombinant DgHBPs and biomimetic peptides were investigated. DgHBP-2 peptide, a biomimetic peptide derived from tandem repeats of DgHBP-2 was found to be most suitable to be used in drug delivery. DgHBP-2 peptide (L-3,4-dihydroxyphenylalanine) DOPA was next synthesized with the incorporation of DOPA, an unnatural amino acid for its catechol moiety. DgHBP-2 peptide and DgHBP-2 peptide DOPA coacervates was crosslinked with different methods and characterized using DLS, electron microscopy, zeta potential and pH stability. *In vitro* cytotoxicity of these coacervates were also done using MTT and live/dead staining to determine its suitability to be used in the fabrication of drug delivery system.

4.2 Experimental Methods

4.2.1 Relative turbidity for coacervation studies

10 μ L of peptide or protein stock solutions (10 mg/mL) was added to 90 μ L of respective buffers to make a final concentration of 1 mg/mL. The level of coacervation at respective buffer conditions was measured at 600 nm using UV-vis spectrophotometry (Lambda 35, Perkin Elmer). Relative turbidity is calculated using the following equation¹⁰:

$$100 - 100 * [10^{-A_{600}}]$$

4.2.2 Optical microscopy

Coacervates were first added to a glass coverslip and then focused under the microscope. Micrographs were taken with an inverted microscope (Axio observer z1, Zeiss) fitted with a 63X oil Immersion lens. Micrographs were processed and exported with Zen imaging (Blue edition, Zeiss) software.

4.2.3 DLS measurements

100 μ L DgHBP-2 peptide coacervates were first pipetted into a disposable cuvette (UVette, Eppendorf) DLS measurements were done on a particle size analyser (ZetaPALs, Brookhaven Instruments Corporation) equipped with a red diode laser (640 nm, 35 mW, 90° scattering angle). Each data was an average of 5 different measurements.

4.2.4 Solid phase peptide synthesis (SPPS)

Two-chlorotriyl chloride resin was swelled in dry Dimethylformamide (DMF) within the reaction vessel for an hour. Ten equivalent of Fluorenylmethyloxycarbonyl (Fmoc)-AA-OH (1st amino acid), 5 equivalent of N,N-Diisopropylcarbodiimide (DIC) and 1 equivalent of 4-Dimethylaminopyridine (DMAP) were dissolved in dry Dichloromethane (DCM). The Fmoc-AA-OH reaction mixture was then stirred for 1 hour at 0°C before adding to the swelled resin. After that, the final Fmoc-AA-OH reaction mixture and resin were incubated overnight.

The unreacted amino acids were washed off with DMF, methanol and DCM. Capping of the unreacted resin was done using DCM, ME OH and DIPEA mixture (85:10:5) for 1 hour. After capping, the beads were washed with DMF, methanol and DCM. Deprotection (cleaving of Fmoc protecting group) was subsequently done in 25% piperidine in DMF for 30 mins. Washing was done 4 times using DMF and DCM to wash off the deprotection reagent. 2,4,6-trinitrobenzene sulfonic acid test (TNBS) was done to determine if the deprotection was completed. These resins would turn from colourless to orange when the deprotection was successful. The second Fmoc-AA-OH (2 equivalent) was coupled onto the resins using 2 equivalent of N,N-Diisopropylethylamine (DIPEA) and 1.9 equivalent of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) for 3 hours. TNBS test was done to ensure the coupling was completed. The resins should remain colourless after coupling. Washing was done 4 times using DMF and DCM to remove unreacted amino acids. The above steps were repeated until all amino acids were added. All the above reactions were done at room temperature and mixed in the presence of nitrogen gas.

Upon completion of the synthesis, the final peptide was cleaved off the resin using 95% Trifluoroacetic acid (TFA) with 2.5% Triisopropylsilyl and 2.5% deionised water for 2 hours. After evaporating the TFA, the peptide was washed and precipitated using diethyl ether at 0°C. Diethyl ether was removed by evaporation and remaining peptide was purified using C18 reverse phase HPLC.

4.2.5 RP-HPLC

DgHBP-2-peptide DOPA synthesised by SPPS method were purified using a C18 reverse phase preparative column (Kromasil). DgHBP-2-peptide DOPA were first dissolved in 7% ACN with 0.1% of TFA and filtered using a 0.2 µm filter. The mobile phases were HPLC grade ACN and deionised water with 0.1% TFA. TFA was added as an additive to prevent ionic interactions and to enhance RP retention. The HPLC machine was purged with 100% ACN at 5 mL/min for 5 mins before the column was attached to the machine. The column was then flushed with 7% ACN at 4 mL/min until the UV absorption reached a steady state. After that, 1 mL of sample was injected into HPLC

machine using a 1 ml sample loop. HPLC protocol: 0 to 15 min → 7 to 30% ACN; 15 to 25 min → 30 to 40% ACN; 25 to 30 min → 40 to 100% ACN; 30 to 35 min → 100% ACN; 35 to 40 min → 100 to 7% ACN. Collection was done between 15 to 25 min with 0.33 min intervals.

4.2.6 Arnow staining

20 μ L of 0.5 M HCL was added to 20 μ L of sample/standard solution. After mixing, 20 μ L of nitrile-molybdate reagent (1:1 sodium nitrite and sodium molybdate) was added to the reaction mixture. 20 μ L of 1M sodium hydroxide was subsequently added to the reaction mixture. Lastly, 20 μ L of deionised water was added to the final reaction mixture. Each reagent was added in the given order and mixing was done after each addition. Absorbance spectrum (300 nm to 700 nm) of the sample/standard was measured using a UV/VIS spectrophotometer. DOPA was used as a positive standard while tyrosine was used as a negative standard.

4.2.7 Maldi-TOF

Sinapinic acid was dissolved in 50% ACN with 0.1% TFA to a concentration of 10 mg/mL. 1 μ L of sinapinic acid was added and dried on the MALDI plate. 1 μ L of sample in 50% ACN with 0.1% TFA (1 mg/mL) was then added on top of the sinapinic acid. After drying, 1 μ L of sinapinic acid was added on top of the sample. The reaction mixture was subsequently air dried before inserting into Shimadzu Biotech TOF machine for analysis. The experiment conditions were as follow: Nitrogen laser was set to 337 nm; Power at 100 W to 120 W; 100 laser shots per analysis; Reflectron mode.

4.2.8 Crosslinking of DgHBP-2 peptide DOPA coacervates

50 μ L of 1 mg/mL DgHBP-2 peptide DOPA coacervates was first diluted to 500 μ L with respective buffer. FeCl_3 or sodium periodate (NaIO_4) was then added to the diluted coacervates to a final concentration of 0.4 mM and 2.4 mM respectively. The final mixture was subsequently vortexed at low speed for 60 s. After crosslinking, the crosslinked coacervates were washed 3 times with deionised water.

4.2.9 Crosslinking of DgHBP-2 peptide coacervates

5 μL of 100 mM 4-MC was added to 50 μL of DgHBP-2 peptide coacervates (1 mg/mL). The coacervates was then diluted with phosphate buffer to final volume of 500 μL . 5 μL of 300 mM NaIO_4 was subsequently added and vortexed promptly for 60 s. After 4 hours of incubation at room temperature, the crosslinked coacervates were washed with deionised water to remove excess 4-MC and NaIO_4 .

4.2.10 Scanning Electron Microscopy (SEM)

Coacervates were first crosslinked and then re-suspend in deionised water after washing. 5 μL of crosslinked coacervates were next air-dried on carbon tape. After drying, crosslinked coacervates were coated with platinum to decrease surface charging. Platinum coating was done with the following conditions (Pressure: 5 Pa, Current: 20 mA and Time: 15 s). Micrographs were taken using a Field Emission Scanning Electron Microscope (7600F, JSM Schottky).

4.2.11 Transmission Electron Microscopy (TEM)

5 μL of crosslinked coacervates were added onto a 400 MESH COPPER TEM grid (01824, Ted Pella) and air-dried overnight. Micrographs were taken using a Transmission Electron Microscope (Libra Plus, Carl Zeiss).

4.2.12 Zeta potential

Zeta potential of coacervates was measured in a zeta potential plastic cell using a nano particle analyser (Sz-100, Horiba). Each sample was measured 3 times and the data was averaged to get the final zeta potential.

4.2.13 MTT assay

Cells were cultured in Dulbecco's modified eagle medium supplemented with 100 units/mL of penicillin, 100 $\mu\text{g/mL}$ of streptomycin and 10% of fetal bovine serum in an incubator with 5% CO_2 at 37°C. 100 μL of cells (concentration: 1×10^4 cells/mL) were added per well of a 96-well plate (Thermo Fisher Scientific). After 24 hours, the cell culture media was substituted with 100 μL of media containing respective concentrations of

coacervates. 10 μ L of MTT reagent (5 mg/mL) was added per well after another 24 hours of incubation. The excess MTT reagent and media were then removed through vacuum aspiration after incubating for 4 hours. 100 μ L of DMSO was next added to each well to dissolve the formazan crystals. The absorbance of the dissolved crystals was measured immediately on a microplate reader (Infinity M200, Tecan) at 570 nm with Native DMSO as a blank. Relative cell viability is calculated using the equation below:

$$(A_t - A_b)/(A_c - A_b) * 100$$

Where A_t , A_b , and A_c designate average absorbance value of wells containing tested cells, native DMSO and control cells correspondingly.

4.2.14 Live/dead staining

Cells were seeded at 5×10^4 cells/mL onto 24-well culture plate (Corning Incorporated) and incubated at 37°C overnight. Cells were next washed using PBS and replaced with culture media containing coacervates. Following 24 hours of incubation at 37°C, cells were washed 3 times with PBS. 2 μ M calcein AM and 4 μ M EthD-1 (LIVE/DEAD® Viability/Cytotoxicity kit, Invitrogen) in PBS was then added to the cells. Imaging was done after 20 min of incubation at room temperature. Micrographs were taken by an inverted microscope (Axio observer z1, Zeiss) equipped with a 20 $\times$ lens

4.3 Characterisation and crosslinking of DgHBP-2 peptide coacervates

4.3.1 Description of tested biomimetic peptides and proteins

A total of five biomimetic peptides and proteins (DgHBP-1 protein, DgHBP-2 protein, pepHBP-1, DgHBP-2-peptide and Mfp3s-peptide) were selected to test whether they are suitable for encapsulation purposes. DgHBP-1 and -2 (Figure 2.1) have been recently successfully expressed in laboratory.^{8, 9} These proteins are thought to undergo coacervation when migrating into the beak's chitin network during beak maturation. Tan and co-workers have shown that recombinant DgHBP-1 protein can undergo simple coacervation.⁸ DgHBP-2 protein is also able to undergo simple coacervation due to sharing

similar tandem repeats as DGHBP-1 protein.⁹ Tan and co-workers also designed a consensus peptide (DgHBP peptide) with five tandem repeats from native DgHBP-2 protein that could also undergo coacervation.⁸ This indicates the importance of the repeats in coacervation. An additional tryptophan has been added to the DgHBP-2 peptide for providing the possibility of functionalization in future experiments (Figure 4.1). PepHBP-1 is a consensus peptide derived from the C-terminal repeats of DgHBP-1 (sequence G113 to Y135).⁹ The main difference between pepHBP-1 and DgHBP-2 was the replacement of two GHGX₁X₂ motifs with GAGFA and GFA motifs (Figure 4.1).

Mussel foot protein 3s (Mf3s) is the most abundant protein found in the adhesive plaques of *Mytilus californianus*.¹⁰ This protein enables adhesion and cohesion to the adhesive plaques and is also able to undergo simple coacervation. A 25-amino acid peptide analogue (Mfp3s-peptide) derived from the Mfp3s has been previously designed by Wei *et al* (Figure 4.1).¹⁰ This biomimetic peptide was designed by following these criteria: retaining all charge and most aromatic residues from native sequence with some flanking residues that have a role in maintaining flexibility and polarity. Due to its similarity to native protein, Mfp3s-peptide has also shown adhesion and phase separation properties.¹⁰

pepHBP-1

GHGLY GAGFA GHGLH GFA GHGLY

DgHBP-2 peptide

GHGVY GHGVY GHGPY GHGPY GHGLY W

Mfp3s-peptide

GYDGY NWPYG YNGYR YGWNK GWNGY

Figure 4.1. Sequence of biomimetic peptides: pepHBP-1, DgHBP-2-peptide and Mfp3s-peptide.

4.3.2 Relative turbidity of biomimetic peptides and recombinant proteins

Coacervation of different biomimetic peptides and recombinant proteins were determined by turbidimetry (Figure 4.2). Optimal coacervation of DgHBP-1 and -2

at 0.1 M ionic strength occurred at pH 6.5 and 7.5 respectively (Figure 4.3A and B). The pH range for DgHBP-1 and -2 coacervation increased with increasing ionic strength because of charge screening. Coacervation of DgHBP-2-peptide occurred within a narrow window of pH 7 to pH 9.5 and there was no difference between the three tested ionic strengths (Figure 4.3C). The level of pepHBP-1 coacervation at lower ionic strengths (0.1 M and 0.5 M) was relatively low (< 50%) whereas the optimum coacervation of pepHBP-1 occurred at pH 8.5 in higher ionic strength (1.0 M and 2.0 M). The Mfp3S-peptide coacervation occurred throughout the different tested pH at high ionic strength (Figure 4.4A).

Figure 4.2. Optical image of plastic cuvettes containing phosphate buffer (left) and DgHBP-2 peptide coacervates (right)

Figure 4.3. Relative turbidity of peptide and protein coacervates (1 mg/mL). (A) DgHBP-1 (B) DgHBP-2 (C) DgHBP-2 peptide (D) pepHBP-1. The turbidity assays were done at 25°C and 5 mins after the onset of coacervation. ($n = 3$, mean $\pm$ S.D.)

Figure 4.4. Coacervation studies of Mfp3s-peptide. (A) Relative turbidity of Mfp3s-peptide coacervates. (B) Micrograph of Mfp3s-peptide coacervates. White arrows represent coacervates

that were attached onto the glass coverslip. The turbidity assays and imaging were done at 25°C and 5 mins after the onset of coacervation. ($n = 3$, mean $\pm$ S.D.)

4.3.3 Microscope images of coacervates

Since turbidimetry only measures the impediment of light, aggregates can also potentially lead to false positive results.¹¹ Hence, microscopy was used to confirm the presence of coacervates. Peptides (pepHBP-1, DgHBP-2-peptide and mfp3S-peptide) and recombinant proteins (DgHBP-1 and DgHBP-2 full-length proteins) coacervates (white arrows) were viewed under inverted light microscope (Figure 4.4B and 4.5). These coacervates were formed at pH 7.5, 0.1 M ionic strength. This condition was chosen because it resembles physiological conditions. The only exception was pepHBP-1 (pH 8.5, 2.0 M ionic strength) and DgHBP-1 full protein (pH 6.5, 0.1 M ionic strength), as they either undergo minimum or no coacervation at pH 7.5. These images confirmed the presence of liquid-like droplets that are the characteristic of coacervates.

Figure 4.5. Micrographs of peptide and protein coacervates (1mg/ml). (A) pepHBP-1 (pH 8.5, 2.0 M ionic strength); (B) DgHBP-2 peptide (pH 7.5, 0.1 M ionic strength); (C) DgHBP-1 protein

(pH 6.5, 0.1 M ionic strength); (**D**) DgHBP-2 protein (pH 7.5, 0.1 M ionic strength). White arrows represent coacervates that were attached onto the glass coverslip. Microscopy of the coacervates was done at 25°C and 5 mins after the onset of coacervation.

Out of the 5 tested peptides and proteins, DgHBP-2-peptide was chosen for future studies. This is because its coacervation occurs at physiological conditions and has minimum aggregation (Table 4.1). DgHBP-2 peptide can be easily modified with unnatural amino acid such as DOPA due to its short sequence, which can be used to endow additional stimuli-responsivity or possibility of crosslinking. DgHBP-2 peptide coacervation occurs within a narrow pH window and hence can be used as pH-sensitive coacervates for controlled drug release.

Table 4.1. Summary of conditions that form coacervation.

Protein/peptide	pH that undergo coacervation				Aggregation
	0.1 M	0.5 M	1.0 M	2.0 M	
DgHBP-1	6.5	6.0 to 6.5	3.5 to 6.5	N/A	No
DgHBP-2	6.0 to 7.5	5.5 to 7.5	3.5 to 7.5	N/A	Yes
pepHBP-1	8.5 to 10	8.5 to 9.5	8.5 to 9.5	8.5 to 9.5	No
DgHBP-2 peptide	7.5 to 9.5	7.0 to 10.0	7.0 to 9.5	N/A	No
Mfp3s-peptide	5.5 to 8.5	3.5 to 8.5	3.5 to 8.5	N/A	Yes

4.3.4 Size of DgHBP-2 peptide coacervates

Coacervates are microdroplets-like and have the ability to merge, forming larger droplets over time.^{12, 13} In order to quantify the size of DgHBP-2 peptide coacervates over time, DLS measurements were performed. As shown in Figure 4.6A, DgHBP-2 peptide had an initial diameter of 8 nm in acetic acid. Upon additional of DgHBP-2 peptide into phosphate buffer (pH 7.5, 0.1M), coacervation occurred and the size of the coacervates were ~800 nm (Figure 4.6B, Time = 0 min). Over time, the coacervates coalescence and the size increased to ~1.6 µm (Figure 4.6B).

Figure 4.6. DLS measurements of DgHBP-2 peptide coacervates over different timepoint. (A) Diameter of DgHBP-2 peptide before coacervation in 10 mM acetic acid. **(B)** Effective diameter of DgHBP-2 peptide coacervates from 0 min to 40 min. ($n = 5$, mean $\pm$ S.D.)

To confirm the coalescence properties of DgHBP-2 peptide coacervates, the coacervates were first loaded with either fluorescein or rhodamine B. These differentially-loaded coacervates were then mixed together. At 0 min, two distinct population of coacervates (with either red or green fluorescence) were observed under fluorescence microscope (Figure 4.7A). Upon incubation at room temperature for 60 mins (Figure 4.7C), most of the coacervates were observed to have yellow fluorescence, indicating that the fluorescein-loaded and rhodamine B-loaded coacervates had merged together. Furthermore, some of the coacervates had both green and red fluorescence (Figure 4.7C, white arrowhead), suggesting that they were still in the process of merging. Size distribution analysis of the coacervates by ImageJ (Figure 4.7B and D) showed that the size of the coacervates decreased, even when merging of the coacervates was confirmed by fluorescence microscopy (Figure 4.7A and C). This discrepancy may be due to the larger coacervates settling down and adhering onto the surface of the Eppendorf tube, such that only smaller coacervates were detected after 60 min of incubation.

Figure 4.7. Merging of fluorescein-loaded and rhodamine B-loaded DgHBP-2 peptide coacervates. (A) Micrograph of the two differentially-loaded coacervates immediately after mixing (0min). White arrows represent coacervates loaded with either RITC or FITC. (B) Size distribution analysis of the coacervates at 0 min by ImageJ software. (C) Micrograph of the two differentially-loaded coacervates after mixing and incubated at room temperature for 60 min. White arrowhead represents coacervates undergoing merging. (D) Size distribution analysis of the coacervates after incubation for 60 min by ImageJ software.

4.3.5 Reversible coacervation of DgHBP-2 peptide

Since coacervation of proteins such as tropoelastin and resilin are known to be reversible^{14, 15}, the reversibility of DgHBP-2 peptide coacervation was investigated by changing the pH of DgHBP-2 coacervates using 0.1 M HCL and NaOH (Figure 4.8). DgHBP-2 coacervates were first allowed to attach onto the glass coverslip ($T = 0$ s). When the pH was decreased below the coacervation pH window by addition of 0.1 M HCl ($T =$

3s), the coacervates started to dissociate ($T = 4.5$ s) and complete dissociation of coacervates was seen at $T = 9$ s). Subsequently, addition of 0.1 M NaOH ($T = 13$ s) to increase the pH back into the coacervation window led to reformation of DgHBP-2 peptide coacervates (Figure 4.8, $T = 43$ s). The coacervates once again settled down and attached to the coverslip at $T = 55$ s).

Figure 4.8. Time-lapse video showing reversible coacervation of DgHBP-2 peptide. DgHBP-2 coacervates were first allowed to attach onto the glass coverslip ($T = 0$ s). Upon addition of 0.1M HCl ($T = 3$ s), the coacervates started to dissociate due to the decrease in pH ($T = 4.5$ s). The coacervates were fully dissociated at $T = 9$ s. Upon addition of 0.1M NaOH to increase the pH ($T = 13$ s), the coacervation process occurred again once the buffer pH was between 7.5 to 9.5 ($T = 43$ s, white arrow). At $T = 55$ s, micrograph of DgHBP-2 showed that the coacervates attached onto the surface of glass coverslips.

One of the best-known proteins that undergoes simple coacervation is tropoelastin and its coacervation is an endothermic process ¹⁴, where heating the buffer increased and induced coacervation. On the other hand, resilin inspired polypeptides undergo simple

coacervation upon cooling.¹⁶ The relationship between buffer temperature and DgHBP-2 peptide coacervation was next explored by cooling or heating the coacervates to different temperature. At temperatures lower than room temperature (5°C to 20°C), the coacervates remained stable with little differences between the tested temperatures. On the other hand, the coacervates started to dissociate with increasing temperature (Figure 4.9). The decrease in level of coacervation was more pronounced at temperatures above 40°C, where a rapid decrease in the turbidity was observed. This is unlike tropoelastin coacervates where heating increases the level of coacervation.

4.3.6 Synthesis of DgHBP-2 peptide DOPA

DOPA is an unnatural amino acid that is similar to tyrosine except that it has an additional hydroxyl group, forming a catechol group. Catechol moiety are common in natural systems and has many important roles such as mussel adhesion and squid beak maturation.^{17, 18} They are extremely reactive and able to interact with both inorganic and organic molecules. Of particular interest is the ability of catechol to undergo crosslinking with metal ions and chemical oxidants.¹⁹ Incorporation of catechol moieties into materials such as polymers for crosslinking has been done in many previous studies.^{17, 20-24} DgHBP-2 peptide DOPA was synthesised using solid phase peptide synthesis chemistry on cleavable chlorotriyl chloride resin. DOPA amino acids replaced all the 5 tyrosine residues during the peptide synthesis process. The peptide was cleaved off from the resins and then stored at -80°C.

Figure 4.9. Stability of DgHBP-2 peptide coacervates (1 mg/mL) at different temperature. ($n = 3$, mean $\pm$ S.D.)

4.3.7 Purification and mass analysis of DgHBP-2 peptide DOPA

DgHBP-2 peptide DOPA was purified by preparative C18 column using RP-HPLC (Figure 4.10A, brown dotted square). The purity of the peptide was 100%, which was confirmed by analytical trace analysis (Figure 4.10B). The molecular weight of DgHBP-2 peptide with different number of DOPA incorporation was shown in Figure 4.10C. MALDI-TOF analysis shown that the molecular weight of DgHBP-2 peptide DOPA is 2866 Da (Figure 4.10D), which corresponded to DgHBP-2 peptide plus five additional hydroxyl groups from the DOPA amino acids..

Figure 4.10. SPPS of DgHBP-2 peptide DOPA. RP-HPLC of DgHBP-2 peptide DOPA by C18 column **(A)** before purification (brown dotted box: peak with desired peptide) **(B)** after purification. **(C)** Theoretical molecular weight of DgHBP-2 peptide DOPA with different number of DOPA amino acids. **(D)** Molecular weight of DgHBP-2 peptide DOPA by MALDI-TOF.

4.3.8 Characterisation of DgHBP-2 peptide DOPA

Catechol moieties undergo spontaneous oxidation upon exposure to light and oxygen.¹⁹ Arnow staining was done to confirm the presence of Catechol moieties.²⁵ In the presence of catechol groups, Arnow staining resulted in a colour change of the peptide solution from colourless to red (Figure 4.11A). The intensity of redness is proportional to the amount of catechol groups within the solution. Arnow staining was quantified by measuring the absorbance between 400-600 nm (Figure 4.11B). The absorbance of DgHBP-2 peptide DOPA and DOPA amino acids (with catechol groups) increased after Arnow staining, whereas there was no absorbance in DgHBP-2 peptide and tyrosine amino acid (without catechol group). The optimum coacervation conditions of DgHBP-2 peptide DOPA was between pH 7 to 9.5 (Figure 4.11C). The ionic strength of the buffer did not

affect the amount of coacervation. This was very similar to DgHBP-2 peptide coacervation conditions. DgHBP-2 peptide DOPA coacervates were viewed under light microscope. Similarly, micrograph of DgHBP-2 peptide DOPA coacervates also showed the presence of liquid-like droplets (Figure 4.11D). These results indicated that the presence of DOPA within the peptide sequence does not affect coacervation process and criteria.

Figure 4.11. Characterisation of DgHBP-2 peptide DOPA. (A) DgHBP-2 peptide DOPA and DgHBP-2 peptide solution after Arnow staining. Colour change is observed only in DgHBP-2 peptide DOPA. (B) Absorbance spectrum of DgHBP-2 peptide DOPA after Arnow staining. Absorbance was increased only in samples that contained catechol groups (DgHBP-2 peptide DOPA and DOPA amino acids). (C) Relative turbidity of DgHBP-2 peptide DOPA coacervates at 1 mg/mL. ($n = 3$, mean $\pm$ S.D.) (D) Micrograph of DgHBP-2 peptide DOPA coacervates at 1 mg/mL.

4.3.9 Crosslinking of DgHBP-2 peptide DOPA coacervates

There are two main catechol crosslinking mechanisms, namely coordination bond and irreversible covalent bond (Figure 4.12). Catechols can binds to transition metals e.g.

Fe to form strong but reversible coordination bonds, resulting in the formation of catechol-metal complexes.¹⁹ Fe was chosen in this study because of its low toxicity and its ubiquitous presence in blood. On the other hand, catechols can be readily oxidised by chemicals e.g. NaIO_4 or enzymes such as tyrosinase to form *o*-quinones. *O*-quinones are extremely reactive and can undergo further secondary reactions with nucleophilic groups such as amine and imidazole to form covalent bonds.¹⁹

Figure 4.12. Illustration of DgHBP-2 peptide DOPA coacervation and subsequent crosslinking.

4.3.10 DOPA-Fe crosslinked coacervates

DgHBpP-2 peptide DOPA was subsequently crosslinked using FeCl_3 and resuspended in deionised water. Micrograph of the DOPA-Fe crosslinked coacervates had shown they were droplets-like sphere which was like the un-crosslinked coacervates (Figure 4.13A). The colour of the coacervates changed from white to blue, which was indicative of catechol-Fe coordination complex. DOPA-Fe crosslinked coacervates are polydisperse (Figure 4.13B), consisting of two coacervates population of different size (~ 300 nm and ~ 1.3 μm). Since the formation of coordination complex is dependent on Fe,

Ethylenediaminetetraacetic acid (EDTA) was used to determine the presence of this complex. EDTA is a metal chelating chemical that binds and holds onto metal ions.²⁶ Upon addition of 25 mM EDTA, the previously stable DOPA-Fe crosslinked coacervates collapsed in water and irregular aggregates were seen instead of spherical droplets (Figure 4.13C). As DOPA-Fe coordination bonds are known to be pH sensitive,²⁷ DOPA-Fe crosslinked coacervates were next subjected to phosphate buffers of different pH. The crosslinked coacervates dissociated at $\text{pH} \leq 4.5$ and $\text{pH} \geq 10$ (Figure 4.13D and E), indicating that they were only stable between pH 5 and 9.5 SEM micrographs showed polydispersity in size distribution between the DOPA-Fe crosslinked coacervates (Figure 4.14A and C, red and white arrows). The surface morphology of these crosslinked coacervates under SEM was smooth and consistent throughout (Figure 4.14B). Higher magnification of DOPA-Fe crosslinked coacervates under TEM has shown that they were uniformly dense (Figure 4.14 D).

Figure 4.13. DgHBP-2 peptide DOPA-Fe crosslinked coacervates. (A) Micrograph (left) and optical image of plastic cuvette (right) containing DOPA-Fe crosslinked coacervates. (B) DLS of DOPA-Fe crosslinked coacervates. (C) Upon additional of 25 mM EDTA to the DOPA-Fe crosslinked coacervates, the coacervates collapsed immediately and only aggregates were observed in the micrograph. (D-E) DOPA-Fe crosslinked coacervates were next subjected to phosphate

buffers of different pH. At $\text{pH} \leq 4.5$ (D) and $\text{pH} \geq 10$ (E), the crosslinked coacervates dissociated, indicating that they are only stable at pH between 5 to 9.5. The EDTA and pH experiments were done at 25°C.

Figure 4.14. Electron microscopy of DOPA-Fe crosslinked coacervates. (A, B) SEM micrographs. (C, D) TEM micrographs. (B and D were electron micrographs of DOPA-Fe crosslinked coacervates taken at a higher magnification in SEM and TEM respectively. White and red arrows represent coacervates of different sizes. The web-like net structure (green arrows) seen under the coacervates in the TEM micrographs is the lacey carbon support film that is part of the TEM copper grids.

4.3.11 DOPA- NaIO_4 crosslinked coacervates

Next, DgHBP-2 peptide DOPA was crosslinked using a common chemical oxidant, NaIO_4 . Micrograph of the DOPA- NaIO_4 crosslinked coacervates had shown that these coacervates were like the un-crosslinked coacervates (Figure 4.15A). The colour of the coacervates changed from white to orange. Similar to DOPA-Fe crosslinked coacervates, DOPA- NaIO_4 crosslinked coacervates were also polydisperse (Figure 4.15B) except that they were slightly larger (~ 400 nm and ~ 1.4 μm). This covalent crosslinking is dependent

on the formation of quinones through oxidation of catechol and is irreversible. Since no metal ions are involved, EDTA did not have any effect on these crosslinked coacervates (Figure 4.15C). Unlike un-crosslinked and DOPA-Fe crosslinked coacervates, DOPA- NaIO_4 crosslinked coacervates were stable in extreme pH (pH 1 and 13, Figure 4.15D and E). SEM micrographs show that the size distribution of DOPA- NaIO_4 crosslinked coacervates was also polydisperse (Figure 4.16A and C, red and white arrow). Other than having a smooth surface, white dots were observed on the surface of these crosslinked coacervates (Figure 4.16B, blue arrow). TEM micrographs of DOPA- NaIO_4 crosslinked coacervates were darker compared to DOPA-Fe crosslinked coacervates, indicating that the DOPA- NaIO_4 crosslinking method resulted in denser coacervates (Figure 4.16C and D).

Figure 4.15. DgHBP-2 peptide DOPA- NaIO_4 crosslinked coacervates. (A) Micrograph (left) and optical image of plastic cuvette (right) containing DOPA- NaIO_4 crosslinked coacervates. (B) DLS of DOPA- NaIO_4 crosslinked coacervates ($n = 5$) (C) Upon additional of 25 mM EDTA to the DOPA- NaIO_4 crosslinked coacervates, the crosslinked coacervates remained stable as observed in the micrograph. (D-E) DOPA- NaIO_4 crosslinked coacervates were next subjected to buffers of different pH (1 to 13). The crosslinked coacervates remained stable at all tested buffers. The EDTA and pH experiments were done at 25°C.

Figure 4.16. Electron microscopy of DOPA-NaIO₄ crosslinked coacervates. (A, B) SEM micrographs. (C, D) TEM micrographs. (B and D were electron micrographs of DOPA-NaIO₄ crosslinked coacervates taken at a higher magnification in SEM and TEM respectively. White and red arrows represent coacervates of different sizes, whereas blue arrows indicate the white spots seen on the surface of crosslinked coacervates. The web-like net structure (green arrows) seen under the coacervates in the TEM micrographs is the lacey carbon support film that is part of the TEM copper grids.

4.3.12 Crosslinking of DgHBP-2 peptide coacervates

DgHBP-2 peptide can also be crosslinked through reactions between individual amino acids residues such as dityrosine crosslinking or with the addition of exogenous crosslinkers such as 4-methyl catechol (Figure 4.17).

Figure 4.17. Illustration of DgHBP-2 peptide coacervation and subsequent crosslinking.

4.3.13 Dityrosine crosslinked coacervates

Since DgHBP-2 peptide has high percentage of tyrosine residues (19.2%), it is possible to stabilise the coacervates through dityrosine crosslinking. Proteins stabilised through dityrosine crosslinking are common in nature such as resilin from desert locust and connective tissues in human aorta.^{28, 29} Dityrosine crosslinking is initiated by the removal of hydroxyl group's hydrogen atom on the phenoxy ring to generate a free tyrosyl radical. This radical then subsequently react with another nearby tyrosyl radical to form dityrosine.²⁸ There are many different reactions to induce dityrosine crosslinking, including enzymatic or photoinitiated reactions. Out of the different enzymes and chemicals used for initiating dityrosine crosslinking, riboflavin is noteworthy due to its low toxicity and because it is found naturally in tissues.²⁸

10 µg/ml of riboflavin was added to DgHBP-2 peptide coacervates and then exposed to UV light for 1 to 6 mins. The level of dityrosine residue was determine by excitation at 315 nm and measurement of its fluorescence at 400nm. The amount of dityrosine residues increased with increasing duration of exposure to UV light (Figure 4.18). Despite the presence of dityrosine crosslinking, the coacervates dissociated upon resuspending in

water, signifying that dityrosine crosslinking was not sufficient for stabilising the coacervates.

Figure 4.18. Dityrosine crosslinking of DgHBP-2 peptide using riboflavin as a photoinitiator. (A) Fluorescence analysis of dityrosine crosslinked peptide. (B) Micrograph of dityrosine crosslinked peptide coacervates in deionised water after centrifugation.

4.3.14 4-Methyl catechol (4-MC)/NaIO₄ crosslinked coacervates

The use of 4-MC as an external crosslinker were next tested for the crosslinking of DgHBP-2 peptide coacervates. It is a chemical compound with two catechol moieties, which can be further oxidised to *o*-quinones by a chemical oxidant such as NaIO₄. The resulting 4-Methyl-1,2-benzoquinone (4-MB) then reacts with nucleophilic groups like primary amines and imidazole nitrogen of histidine.

Micrograph of the 4-MC/NaIO₄ crosslinked coacervates had revealed that these coacervates were yellow and have droplet-like morphology (Figure 4.19A). These coacervates also had the tendency to sticking to each other. The size of 4-MC/NaIO₄ crosslinked coacervates were smaller (~160 nm and ~800 nm) compared to the previous DOPA crosslinked coacervates. (Figure 4.19B). SEM and TEM micrographs confirmed that two different size population of 4-MC/NaIO₄ crosslinked coacervates were generated during the crosslinking process (Figure 4.20A and C, red and white arrow). Like DOPA-Fe crosslinked coacervates, these coacervates had a smooth surface and were highly dense (Figure 4.20C). Higher magnification of TEM macrographs showed a “budding” effect (Figure 4.20D, purple arrow), suggesting that the surface of the crosslinked coacervates

was dynamic and the smaller coacervates population (~ 160 nm) could be generated by this “budding” process (Ostwald ripening).

Figure 4.19. DgHBP-2 peptide coacervates crosslinked with 4-MC and NaIO₄. (A) Micrograph (left) and optical image of plastic cuvette (right) containing 4-MC/NaIO₄ crosslinked coacervates. (B) DLS of 4-MC/NaIO₄ crosslinked coacervates. ($n = 5$)

Figure 4.20. Electron microscopy of 4-MC/NaIO₄ crosslinked coacervates. (A, B) SEM micrographs. (C, D) TEM micrographs. (B and D were electron micrographs of 4-MC/NaIO₄ crosslinked coacervates taken at a higher magnification in SEM and TEM respectively. White and red arrows represent coacervates of different sizes, whereas purple arrow indicates the budding

effect from the crosslinked coacervates. The webbed-like net structures (green arrows) seen under the coacervates in the TEM micrographs are the lacey carbon support film that are part of the TEM copper grids.

4.3.15 Zeta potential of DgHBP-2 coacervates.

Zeta potential is a measure of the charge that forms on the boundary between particle surface and its buffer medium. Since zeta potential can have an impact on the pharmacokinetic properties or cell uptake of coacervates, zeta potential measurements were done both the un-crosslinked and crosslinked coacervates (Figure 4.21). DgHBP-2 peptide coacervates had a positive zeta potential of 1.5 mV whereas zeta potential became negative upon crosslinking (DOPA-Fe: -10.5 mV; DOPA-NaIO₄: -0.3 mV and 4-MC/NaIO₄: -9.0 mV).

Figure 4.21. Zeta potential measurement of DgHBP-2 peptide coacervates. (A) phosphate buffer (pH 7.5, 0.1M) (B) PBS (C) DgHBP-2 peptide coacervates (D) DOPA-Fe crosslinked coacervates (E) DOPA-NaIO₄ crosslinked coacervates (F) 4-MC/NaIO₄ crosslinked coacervates. ($n = 5$)

4.3.16 *In vitro* cytotoxic assay on NIH3T3 fibroblasts

After completing the above characterisation studies, MTT assay was done on NIH3T3 mouse fibroblasts to study the toxicity of DgHBP-2 peptide coacervates. MTT assay evaluates cell viability by measuring metabolic rate of the tested cells. From Figure 4.22, the relative cell viabilities for DgHBP-2 peptide and crosslinked coacervates treated fibroblasts were at least 90%. These data indicated that the peptide and coacervates were not toxic to the cells.

Figure 4.22. Relative cell viability of NIH3T3 mouse fibroblast treated with (A) DgHBP-2 peptide, (B) DOPA-Fe crosslinked coacervates, (C) DOPA-NaIO₄ crosslinked coacervates, and (D)

4-MC/NaIO₄ crosslinked coacervates. Control cells (without treatment) were normalised to 100%. ($n = 6$, mean $\pm$ S.D.)

In addition to MTT assay, live/dead staining was also done to determine the toxicity of DgHBP-2 peptide coacervates. Live/dead staining directly measures the physical and biochemical activity of the cells and complement the results generated from MTT assay. Viable cells hydrolyse calcein AM that is non-fluorescence to calcein that is green fluorescence whereas ethidium homodimer-1 binds to DNA of dead cells and emit red fluorescence (Figure 4.23A and B). From the fluorescence micrographs (Figure 4.23C to F), majority of cells are emitting mostly green fluorescence and minimum red fluorescence. This was in consistent with results from MTT assays, where DgHBP-2 peptide coacervates and crosslinked coacervates was non-toxic to NIH3T3 fibroblasts.

Figure 4.23. Live/dead staining of NIH3T3 fibroblast cells treated with coacervates. (A) Control live cells, (B) control dead cells (killed by 70% methanol), (C) DgHBP-2 peptide coacervates, (D) DOPA-Fe crosslinked coacervates, (E) DOPA-NaIO₄ crosslinked coacervates, and (F) 4-MC/NaIO₄ crosslinked coacervates.

4.4 Conclusions

In this chapter, the optimum coacervation conditions for different recombinant proteins and biomimetic peptides were first compared using turbidimetry and light microscopy. After comparison, DgHBP-2 peptide was determined to be best suited as an

encapsulation material due to its lack of aggregates and coacervation pH range (7.5 to 9.5). The size of DgHBP-2 peptide in acetic acid was 8 nm, coacervation occurred upon addition of the peptide to phosphate buffer and the size increases to 800 nm. The coacervates coalesced over time and the size increased to ~1.6 μm . This coacervation process was reversible by changing the pH. Unlike tropoelastin coacervation, DgHBP-2 peptide coacervates was stable in temperatures between 5°C to 25°C and dissociated rapidly when the temperature increased to beyond 40°C. These results have shown that DgHBP-2 peptide is both pH and temperature responsive, making it useful as an environmental responsive encapsulation material.

Endogenous catechol moieties (DOPA) or exogenous catechol moieties (4-MC) with either NaIO_4 or FeCl_3 were used to crosslink coacervates for the purpose of stabilising them. Electron microscopy shown that these coacervates had smooth surface features and were highly dense. In addition, these crosslinked coacervates were cytocompatible, making them ideal to be used as drug delivery systems.

The first objective of this thesis, whether DgHBP based coacervates are suitable to be used in drug delivery system was answered in this chapter. The results from the experiments done in this chapter allowed us to understand and better design future delivery systems. With these results in mind, the development and fabrication of ‘smart’ delivery systems would be detailed in the next two chapters.

Reference

1. N. R. Johnson and Y. Wang, *Expert Opin Drug Deliv*, 2014, **11**, 1829-1832.
2. A. C. Obermeyer, C. E. Mills, X. H. Dong, R. J. Flores and B. D. Olsen, *Soft Matter*, 2016, **12**, 3570-3581.
3. A. Perro, L. Giraud, N. Coudon, S. Shanmugathasan, V. Lapeyre, B. Goudeau, J. P. Douliez and V. Ravaine, *J Colloid Interface Sci*, 2019, **548**, 275-283.
4. J. M. Horn, R. A. Kapelner and A. C. Obermeyer, *Polymers (Basel)*, 2019, **11**, 578.
5. M. A. Cruz, D. L. Morris, J. P. Swanson, M. Kundu, S. G. Mankoci, T. C. Leeper and A. Joy, *ACS Macro Letters*, 2018, **7**, 477-481.

6. B. Vrhovski, S. Jensen and A. S. Weiss, *Eur J Biochem*, 1997, **250**, 92-98.
7. N. K. Li, F. Garcia Quiroz, C. K. Hall, A. Chilkoti and Y. G. Yingling, *Biomacromolecules*, 2014, **15**, 3522-3530.
8. Y. Tan, S. Hoon, P. A. Guerette, W. Wei, A. Ghadban, C. Hao, A. Miserez and J. H. Waite, *Nat Chem Biol*, 2015, **11**, 488-495.
9. H. Cai, B. Gabryelczyk, M. S. S. Manimekalai, G. Gruber, S. Salentinig and A. Miserez, *Soft Matter*, 2017, **13**, 7740-7752.
10. W. Wei, L. Petrone, Y. Tan, H. Cai, J. N. Israelachvili, A. Miserez and J. H. Waite, *Adv Funct Mater*, 2016, **26**, 3496-3507.
11. D. Hall, R. Zhao, I. Dehlsen, N. Bloomfield, S. R. Williams, F. Arisaka, Y. Goto and J. A. Carver, *Anal Biochem*, 2016, **498**, 78-94.
12. K. K. Nakashima, J. F. Baaij and E. Spruijt, *Soft Matter*, 2018, **14**, 361-367.
13. S. Deshpande, F. Brandenburg, A. Lau, M. G. F. Last, W. K. Spoelstra, L. Reese, S. Wunnava, M. Dogterom and C. Dekker, *Nat Commun*, 2019, **10**, 1800.
14. G. C. Yeo, F. W. Keeley and A. S. Weiss, *Adv Colloid Interface Sci*, 2011, **167**, 94-103.
15. L. Li, T. Luo and K. L. Kiick, *Macromol Rapid Commun*, 2015, **36**, 90-95.
16. R. Balu, J. Whittaker, N. K. Dutta, C. M. Elvin and N. R. Choudhury, *J. Mater. Chem. B*, 2014, **2**, 5936-5947.
17. A. Andersen, Y. Chen and H. Birkedal, *Biomimetics (Basel)*, 2019, **4**.
18. M. Yu, J. Hwang and T. J. Deming, *Journal of the American Chemical Society*, 1999, **121**, 5825-5826.
19. J. Yang, M. A. Cohen Stuart and M. Kamperman, *Chem Soc Rev*, 2014, **43**, 8271-8298.
20. J. H. Ryu, Y. Lee, W. H. Kong, T. G. Kim, T. G. Park and H. Lee, *Biomacromolecules*, 2011, **12**, 2653-2659.
21. Z. Wang, S. Zhao, R. Song, W. Zhang, S. Zhang and J. Li, *Sci Rep*, 2017, **7**, 9664.
22. X. Liu, J. Deng, L. Ma, C. Cheng, C. Nie, C. He and C. Zhao, *Langmuir*, 2014, **30**, 14905-14915.
23. Z. Zeng and X. Mo, *J Biomater Appl*, 2017, **32**, 612-621.

24. A. Khan, M. Xu, T. Wang, C. You, X. Wang, H. Ren, H. Zhou, A. Khan, C. Han and P. Li, *Biosci Rep*, 2019, **39**, BSR20190504.
25. S. Spaans, P. Fransen, B. D. Ippel, D. F. A. de Bont, H. M. Keizer, N. A. M. Bax, C. V. C. Bouten and P. Y. W. Dankers, *Biomater Sci*, 2017, **5**, 1541-1548.
26. S. J. Flora and V. Pachauri, *Int J Environ Res Public Health*, 2010, **7**, 2745-2788.
27. H. Zheng, L. Xing, Y. Cao and S. Che, *Coordination Chemistry Reviews*, 2013, **257**, 1933-1944.
28. B. P. Partlow, M. B. Applegate, F. G. Omenetto and D. L. Kaplan, *ACS Biomaterials Science & Engineering*, 2016, **2**, 2108-2121.
29. S. O. Andersen, *Insect Biochem Mol Biol*, 2004, **34**, 459-466.

Chapter 5

Glucose responsive insulin delivery system

This chapter presents the fabrication of a 'smart' insulin delivery system, made from DgHBP-2 peptide coacervates derived from the beak protein of Dosidicus Gigas. In this delivery system, GOx and insulin were both encapsulated within the peptide coacervates. GOx acted as a glucose sensor, responding to increasing glucose levels by creating a local acidic environment, which resulted in the dissociation of coacervates and finally release of insulin cargo. This chapter presents the results relating to cytotoxicity, encapsulation efficiency of insulin, insulin release kinetics in different glucose concentrations, pulsatile release of insulin and the secondary structure of insulin after its was released. The results showed that the rate of insulin release was dependent on the level of glucose, decreases in normoglycemic levels and increases in hyperglycaemic levels. Also, secondary structure of released insulin was found to be unaffected by the encapsulation and release process, indicating the retention of its bioactivity.

This section published substantially as Zhi Wei Lim, Yuan Ping, Ali Miserez, *Glucose-Responsive Peptide Coacervates with High Encapsulation Efficiency for Controlled Release of Insulin*, Bioconjugate Chemistry 2018 29 (7), 2176-2180.

5.1 Introduction

Diabetes is a deadly, chronic metabolic disease where the patient has high fasting blood glucose levels. Currently, insulin is frequently administered subcutaneously to regulate the blood glucose level in most diabetic patients, but many side effects have been associated with this method of delivery.¹ To obtain optimum therapeutic outcome, patients must also adhere to a strict insulin administration program. Also, the amount of insulin needed is dependent on many factors such as patient's activities and amount of food intake. Thus, patients can easily suffer from side effects due to incorrect insulin dosage. Therefore, many researches have been done to fabricate 'smart' or environmentally responsive delivery system.²

A GRIDS can sense the surrounding glucose and then release insulin accordingly to the glucose level.³⁻¹³ GRIDS can replace recurring insulin injection and increases the accuracy of insulin dosage by lessening the degree of patient's direct participation in glucose management.^{1, 3, 5, 6, 8-10, 13} One common method used for detecting glucose is through the incorporation of a glucose responsive element like GOx and boronic acid into the delivery system.¹⁴⁻¹⁷ GOx converts glucose into gluconic acid, resulting in a localised decrease in pH. The acidic pH consequently triggers the structural or conformational changes in the materials of the insulin carrier and finally result in the release of insulin.¹⁸⁻³³ Such a glucose responsive and insulin delivery strategy was notably used in pH responsive hydrogels.^{19, 20, 22, 23, 29, 30}

Since coacervates are capable of encapsulating both high and low molecular weight therapeutics and coacervation of DgHBP-2 peptide is pH sensitive,^{34, 35} DgHBP-2 peptide coacervates can be used to create GRIDS by encapsulating both GOx and insulin. In this GRIDS, the coacervates function as an insulin reserve whereas GOx induced insulin release from coacervates upon glucose exposure (Figure 5.1A). This triggered release was due to conversion of glucose to gluconic acid which in turn dissociate the coacervates and release of insulin (Figure 5.1B).

Figure 5.1. Illustration of GRIDS created from DgHBP-2 peptide coacervates. (A) Coacervation and encapsulation of insulin and GOx. **(B)** Once the insulin +GOx loaded coacervates are subjected to glucose, the glucose enters the coacervates and hydrolyses to gluconic acid. This leads to a localised pH decrease within the coacervates, which subsequently cause the coacervates to dissociate and releases the insulin.

5.2 Experimental methods

5.2.1 Labelling of Insulin and GOx

Fluorescein isothiocyanate (FITC) and Rhodamine B isothiocyanate (RITC) was first dissolved in acetone to a stock solution of 3 mg/mL. Two hundred microliter of stock dye solution was added drop by drop to 1 mg of GOx or insulin dissolved in 0.1 M carbonate buffer (pH 8.5) or 0.1 M phosphate buffer (pH 7) respectively. The mixture was

then incubated in the dark for 16 hours at room temperature with constant stirring. Excess unreacted dye was removed using a gel filtration column (PD-10, GE healthcare life sciences). Phosphate buffer was then used to elute the labelled GOx and insulin from the column.

5.2.2 MALDI-TOF

The experimental method used in this section was same as in chapter 4.2.7

5.2.3 Drug Loading into coacervates

Insulin and/or GOx were added to phosphate buffer before coacervation to their final concentrations. Peptide stock solution (10 mg/mL) was successively added to the buffer+drug mixture in a 1:9 ratio to trigger encapsulation and coacervation. Pipetting was promptly done to mix the final mixture.

5.2.4 Fluorescence and optical microscopy

Micrographs were taken by an inverted microscope (Axio observer z1, Zeiss) equipped with a 63X oil Immersion lens. Micrographs were processed with Zen imaging (Blue edition, Zeiss) and Photoshop (Adobe) software.

5.2.5 Fluorescence emission assay for insulin-FITC

Insulin-FITC spectra was measured using spectrofluorometer (Fluorolog, Horiba). Insulin-FITC was excited at 490 nm and its fluorescence spectra was measured from 500 nm to 550 nm. A quartz cuvette was used for measuring the fluorescence.

5.2.6 Encapsulation efficiency (Fluorescence method)

Insulin loaded coacervates were removed by centrifugation at a speed of 13k rpm for 5mins. The supernatant was then collected for fluorescence measurement (Fluorolog, HORIBA Jobin Yvon). The concentration of insulin within the supernatant was back-calculated using a standard curve. The EE% is calculated with the equation below:

$$EE\% = \frac{\text{Total Insulin} - \text{Free Insulin}}{\text{Total Insulin}} \times 100\%$$

Where Total Insulin represents the initial Drug concentration and Free Insulin represents the concentration of unencapsulated Insulin measured within supernatant.

5.2.7 Encapsulation efficiency (RP-HPLC method)

Insulin-loaded coacervates were centrifuged at 13K rpm for 5mins. The supernatant was subsequently diluted to 5% ACN. The pellet was also solubilised in 5% ACN. Both supernatant and pellet were analysed with an analytical column (C18, Agilent). HPLC settings: 0-25 min, 5% to 40% ACN; 25-30 min, 40% to 100% ACN; 30-35 min, 100% ACN; 35-40 min, 100% to 5% ACN. Flow rate of 1 mL/min and injection volume of 50 μ L.

5.2.8 MTT assay

The experimental method used in this section was same as in chapter 4.2.13

5.2.9 *In vitro* release assay (Glucose responsive time point assay)

50 μ L of insulin-FITC (0.1mg/mL) and/or GOx (0.01 mg/mL) loaded DgHBP-2 peptide coacervates (1 mg/mL) were diluted with phosphate buffer (0.1M, pH 7.5) to a final volume of 150 μ L. The diluted coacervates were then pipetted into respective dialysis tubes of 12 to 14 kDa cut-off (D-Tube Dialyzer Mini, Millipore). The coacervates was subsequently dialysed against phosphate buffer, with/without respective concentrations of glucose for 48 hours at 37°C (speed: 250 rpm). The dialysed buffer was replaced with new buffer at respective time points and the former was analysed by a fluorescence spectrophotometer (Fluorolog, HORIBA Jobin Yvon).

5.2.10 *In vitro* release assay (alternating glucose concentration assay)

50 μ L of 1 mg/mL of DgHBP-2 peptide coacervates were loaded with 0.1 mg/mL of insulin-FITC and 0.01 mg/mL of GOx. The coacervates was then dialysed against phosphate buffer with glucose (1 or 4 mg/mL) for 9 hours at 37°C (speed: 250 rpm).

Glucose concentration was interchanged between 1 and 4 mg/mL every 90 mins. The dialysed buffer was then measured for its fluorescence using a fluorescence spectrophotometer (Fluorolog, HORIBA Jobin Yvon) at every 90 mins.

5.2.11 Circular Dichroism (CD) Spectroscopy

Native insulin released insulin and DgHBP-2 peptide were diluted to 0.1 mg/mL and 1 mg/mL respectively. CD spectra measurements were analysed using a quartz cuvette of either 0.2 or 0.5 mm optical path length. Data collection was performed on a CD spectrometer (AVIV 420, Aviv Biomedical) with the following parameters: average of 3 scans per sample (wavelength range between 190 to 250 nm), 1.0 nm wavelength steps (0.1s averaging time and 1.00 nm bandwidth). Each sample was repeated for 3 times and averaged to obtain the final CD spectrum. OriginPro 9.1 (OriginLab) was then used to plot and smoothened the final CD spectrum.

5.2.12 Enzyme-linked Immunosorbent Assay (ELISA)

100 μ L of released insulin and native insulin were added to appropriate wells (Human Insulin ELISA kit, Abcam). The plate was then covered and incubated at room temperature with gentle shaking for 2.5 hour. The solution was discarded and the wells were wash 4 times with 1X wash solution. The plate was subsequently blotted against clean towels to remove any remaining wash buffer. 100 μ L of 1X biotinylated insulin antibody was added to each well and incubated with gentle shaking at room temperature for 1 hour. Excess antibody was discarded and the wells were wash 4 times with 1X wash solution. 100 μ L of 1X HRP-streptavidin solution was next added to each well and incubated with gentle shaking at room temperature for 45 min. The wells were wash 4 times with 1X wash solution to remove excess HRP-streptavidin. 100 μ L of TMB One-Step Substrate reagent was added to each well and incubated in the dark at room temperature for 30 min. 50 μ L of stop solution was added to each well and absorbance of the ELISA plate was measured at 450 nm immediately.

5.3 Insulin and GOx-loaded coacervates

5.3.1 Labelling and encapsulation of insulin-FITC

For easy visualisation of insulin under microscope, insulin was labelled with FITC. After labelling, insulin-FITC was purified to remove unreacted FITC and subsequently molecular weight of insulin-FITC was measured. Molecular weight obtained with Maldi-TOF had shown that the majority of human insulin had been labelled with one FITC molecule, whereas the rest were labelled with two FITC molecules and native insulin (Figure 5.2A). DgHBP-2 peptide coacervates were able to encapsulate insulin-FITC, resulting in higher and localised fluorescence intensity (Figure 5.2B). The low background signal also showed that most of the insulin-FITC have been loaded into the coacervates.

Figure 5.2. Purification of insulin-FITC and its encapsulation by DgHBP-2 peptide coacervates. (A) Maldi-TOF results of human insulin labelled with FITC. (B) Micrographs of DgHBP-2 peptide coacervates (1 mg/mL) loaded with insulin-FITC (0.1 mg/mL). Light micrograph (left); fluorescence micrograph (middle); merged micrograph (right). The green fluorescence and grey droplets were insulin-FITC and DgHBP-2 peptide coacervates respectively.

5.3.2 Encapsulation efficiency of insulin-FITC by DgHBP-2-peptide coacervates

The EE% by 1 mg/mL of DgHBP-2 was >99% in all tested insulin-FITC concentrations (0.01-0.4 mg/mL) (Table 5.1). Since the EE% was already >99%, any increased in concentration of DgHBP-2 peptide did not lead to further increase in efficiency. The EE% decreases slightly when the concentration of peptide was below 0.3 mg/ml (Table 5.2). High EE% is expected, as coacervation is known to have high loading capacity. This indicates that more insulin can be loaded within each coacervate and lesser wastage during the loading process. This high EE% was further verified by RP-HPLC on native insulin loaded coacervates. Native insulin was observed only in the dissolved coacervates pellet (Figure 5.3D) and not supernatant (Figure 5.3C), indicating that most native insulin was encapsulated within coacervates. This verified that encapsulation of insulin occurs irrespective of FITC functionalization.

Table 5.1. EE% of insulin-FITC by 1 mg/mL of DgHBP-2-peptide coacervates. (*n* = 3, mean values $\pm$ S.D)

FITC-Insulin (mg/mL)	DgHBP-2 peptide (mg/mL)	Encapsulation efficiency (%)
0.01	1.0	99.7 $\pm$ 0.08
0.05	1.0	99.8 $\pm$ 0.05
0.10	1.0	99.8 $\pm$ 0.02
0.20	1.0	99.7 $\pm$ 0.02
0.40	1.0	99.3 $\pm$ 0.07

Table 5.2. EE% of 0.1 mg/mL of insulin-FITC by DgHBP-2-peptide coacervates. ($n = 3$, mean values $\pm$ S.D)

FITC-Insulin (mg/mL)	DgHBP-2 peptide (mg/mL)	Encapsulation efficiency (%)
0.1	0.1	45.0 $\pm$ 2.97
0.1	0.3	94.4 $\pm$ 0.42
0.1	0.5	99.0 $\pm$ 0.12
0.1	1.0	99.8 $\pm$ 0.02
0.1	2.0	99.9 $\pm$ 0.01

Figure 5.3. RP-HPLC absorbance chromatograms of (A) DgHBP-2 peptide in 10 mM acetic acid (1 mg/mL); (B) native insulin (0.1 mg/mL); (C) supernatant after removing the insulin-loaded coacervates; and (D) dissolved coacervates pellet after removing supernatant. Red arrow specifies

the retention time of native insulin. After insulin encapsulation, virtually no insulin was detected in the supernatant, whereas after dissolving the coacervates pellet, insulin was detected within the coacervates. These data further confirmed the high EE% of insulin by coacervates as measured by fluorescence spectroscopy (Table 5.1 and 5.2).

5.3.3 GOx for sensing glucose

GOx is an enzyme that catalyses the oxidation of glucose into glucolactone, which eventually hydrolyses into gluconic acid. This decrease in pH from gluconic acid is then used to indicate the presence of glucose. In combination with DgHBP-2 peptide coacervates which are stabilised at pH > 7, it can become a glucose and pH-sensitive vehicle for controlled insulin release (Figure 5.1). Next, co-encapsulation of GOx and insulin was tested on DgHBP-2 peptide coacervates. GOx and insulin was first labelled with RITC and FITC respectively for visualisation under fluorescence microscope. The co-localisation of red fluorescence (GOx-RITC) and green fluorescence (insulin-FITC) to yellow fluorescence showed that they can be concomitantly loaded into the coacervates (Figure 5.4).

Figure 5.4. Micrographs of DgHBP-2 peptide coacervates (1 mg/mL) loaded with GOx-RITC (0.1 mg/mL) and insulin-FITC (0.1 mg/mL). (A) Green fluorescence micrograph represents insulin-FITC. (B) Red fluorescence micrograph represents GOx-RITC. (C) Light microscopy micrograph represents DgHBP-2 peptide coacervates. (D) Merged (green, red, and light) micrograph. Yellow fluorescence within coacervates confirmed co-encapsulation of GOx-RITC and insulin-FITC.

5.3.4 In vitro cytotoxic assay on NIH3T3 fibroblasts

MTT assay was done on insulin loaded DgHBP-2 peptide coacervates to study its toxicity on NIH3T3 fibroblast. From Figure 5.5, The relative cell viability of DgHBP-2 peptide coacervates and insulin-loaded coacervates were close to 100%, demonstrating that toxicity of these coacervates was insignificant at the tested concentrations.

Figure 5.5. Relative cell viability of coacervates treated NIH3T3 fibroblasts. (A) DgHBP-2 peptide coacervates, and (B) Insulin-loaded DgHBP-2 peptide coacervates. Control cells (without treatment) were normalized to 100%. ($n = 6$, mean $\pm$ S.D.)

5.3.5 In vitro release of insulin triggered by glucose

Next, to study if release of insulin could be triggered from DgHBP-2 peptide coacervates through glucose-sensing, GOx+insulin loaded coacervates (0.01 mg/mL of GOx, 0.1 mg/mL of insulin and 1 mg/mL of DgHBP-2 peptide) and insulin loaded coacervates (0.1 mg/mL of insulin and 1 mg/mL of DgHBP-2 peptide) were prepared in

phosphate buffer and then exposed to 4 mg/mL of glucose. 4 mg/ml of glucose was selected to simulate hyperglycaemia. The release of insulin was much faster in the presence of both glucose and GOx (Figure 5.6, black line) compared to absence of glucose (Figure 5.6, purple line) or GOx (Figure 5.6, pink and red line). This indicated that glucose had diffused in and subsequently converted into gluconic acid within the coacervates by GOx. As gluconic acid built up within the coacervates, local acidification occurred resulting in the dissociation of coacervates. While leakage of insulin was also seen in the absence of glucose, the amount and rate of insulin release from GOx+insulin loaded coacervates were much higher with glucose over the 48-hour period.

After confirming GOx+insulin coacervates were indeed glucose responsive, the ability to react to fluctuations in glucose concentration was examined next. Since blood glucose level fluctuates according to food intake and activities, it is important that GOx+insulin coacervates can alter the rate of insulin release accordingly to the surrounding glucose concentration. GOx+insulin coacervates were placed in phosphate buffer where the concentration of glucose was altered between hyperglycaemic (4 mg/mL) and normoglycemic (1 mg/mL) levels every 1.5 hours (Figure 5.6B). GOx+insulin coacervates exhibited a pulsatile release of insulin where the rate of insulin release increased at hyperglycaemic levels and decreased at normoglycemic concentrations. This glucose responsive insulin release behaviour could be observed for a minimum of 3 cycles, confirming that only a part of the insulin cargo was released at each cycle. This is consistent with the continuous release of insulin seen in Figure 5.6A.

The rate of insulin release at different glucose concentrations was next investigated. GOx+insulin coacervates were placed in phosphate buffer containing different concentrations of glucose (1 mg/mL to 4 mg/mL). The rate of insulin release increased with increasing concentration of glucose as shown in Figure 5.7. This indicated that not only the GOx+insulin coacervates were sensitive to presence of glucose but also to the concentration of glucose.

Figure 5.6. Glucose triggered release of insulin from DgHBP-2 peptide coacervates in phosphate buffer (A) Insulin loaded coacervates with 4 mg/L of glucose (circle, red); insulin loaded coacervates without glucose (inverted triangle, pink); GOx+insulin coacervates without glucose (triangle, blue); and GOx+insulin coacervates with 4 mg/mL of glucose (square, black). **(B)** Alternate hyperglycaemic (4 mg/mL, blue) and normoglycaemic (1 mg/mL, green) solution. ($n = 3$, mean $\pm$ S.D.)

Figure 5.7. *In vitro* release assay of insulin from DgHBP-2 peptide coacervates in different glucose concentration (1 mg to 4 mg). 4 mg/mL of glucose (square, black); 3 mg/mL of glucose (inverted triangle, pink); 2 mg/mL of glucose (triangle, blue); and 1 mg/mL of glucose (circle, red). ($n = 3$, mean $\pm$ S.D.)

5.3.6 Activity of insulin after encapsulation and release

For a GRIDS to be used in the treatment of diabetic patients, the biological activity of insulin must be retained after its encapsulation and subsequent release from the coacervates.³⁴ Circular dichroism (CD) was first used to verify the biological activity of insulin by assessing its secondary structure (Figure 5.8A). CD spectra of native insulin solution exhibited two minima at 208nm and 202nm, which was the classical signature of α -helical structure (Figure 5.8A, green line). After encapsulation and triggered release of insulin, its CD spectra also displayed the classical signature of α -helical structure except for lower intensity (Figure 5.8, red line). This lower intensity can be due to the weaker ellipticity of the DgHBP-2 peptide present within the solution. CD spectra of thermally denatured insulin (Figure 5.8, dotted line) had shown significant differences between the native or release insulin, particularly the loss of the 222 nm minimum and 195 nm maximum, along with a peak shift in the 208 nm minimum towards smaller wavelength. Since encapsulated insulin were released at mild acidic microenvironments, CD spectra of native insulin were further analysed at mild acidic conditions (Figure 5.9). CD spectra of insulin at these acidic conditions were identical to that of physiological pH, indicating that secondary structure of insulin was not affected at these acidic conditions. This strongly suggested that bioactivity of insulin was retained after being released from coacervates.

In addition to probing the secondary structure of insulin, DgHBP-2 peptide/insulin solution was examined using ELISA. Released insulin was detected at levels similarly to native insulin, signifying that insulin antibodies could recognise and bound to both released and native insulin (Figure 5.8). This suggests that the structure of released insulin was likely not affected during the encapsulation and release process. Together, these results are consistent with previous studies showing that therapeutics retain or even display improved bioactivity/stability when encapsulated within the coacervates, and that native proteins are preferably sequestered inside coacervates as compared to their denatured state.³⁶⁻³⁸

Figure 5.8. Comparison of native and released insulin from DgHBP-2 peptide coacervates. (A) CD spectra of DgHBP-2 peptide (black line), released insulin (red line), heat denatured insulin (blue dotted line), and native insulin (Green line). (B) ELISA of released and native insulin. ($n = 3$, mean $\pm$ S.D.)

Figure 5.9. CD spectra of native insulin in mildly acidic buffers (pH 5.5 to 7.5). ($n = 3$)

5.4 Conclusions

In this chapter, a simple yet effective GRIDS based from insulin and GOx loaded DgHBP-2 peptide coacervates was described. This GRIDS was fabricated by mixing DgHBP-2 peptide with phosphate buffer comprising of GOx and insulin. High EE% (>99%) of insulin allows the coacervates to act as an insulin reservoir while the GOx acts as a

glucose sensor. During hyperglycaemia, glucose molecules diffuse into coacervates and hydrolyses into gluconic acid by GOx. As the gluconic acid accumulates, a localised drop in pH will occurs leading to the disassembly of coacervates and ultimately release of insulin. The release rate of insulin can be tuned accordingly to the surrounding glucose levels. Moreover, the secondary structure of insulin is retained even after encapsulation and exposure to acidic microenvironment. The lack of organic solvents during the encapsulation process contributes greatly to the preservation of insulin structure. This novel GRIDS unifies the high loading capacity of coacervates with glucose responsiveness of GOx to be a potential insulin delivery system for diabetes management.

Reference

1. S. Yaturu, *World J Diabetes*, 2013, **4**, 1-7.
2. Z. Liu, X. J. Ju, W. Wang, R. Xie, L. Jiang, Q. Chen, Y. Q. Zhang, J. F. Wu and L. Y. Chu, *Curr Pharm Des*, 2017, **23**, 295-301.
3. D. Elleri, D. B. Dunger and R. Hovorka, *BMC Med*, 2011, **9**, 120.
4. M. Tauschmann, H. Thabit, L. Bally, J. M. Allen, S. Hartnell, M. E. Wilinska, Y. Ruan, J. Sibayan, C. Kollman, P. Cheng, R. W. Beck, C. L. Acerini, M. L. Evans, D. B. Dunger, D. Elleri, F. Campbell, R. M. Bergenstal, A. Criego, V. N. Shah, L. Leelarathna, R. Hovorka and A. P. Consortium, *Lancet*, 2018, **392**, 1321-1329.
5. V. Dadlani, J. E. Pinsker, E. Dassau and Y. C. Kudva, *Curr Diab Rep*, 2018, **18**, 88.
6. P. Brunetti, M. Massi Benedetti, G. Calabrese and G. P. Reboldi, *Int J Artif Organs*, 1991, **14**, 216-226.
7. J. Y. Stone, N. Haviland and T. S. Bailey, *Ther Deliv*, 2018, **9**, 77-87.
8. A. R. Kahkoska, E. J. Mayer-Davis, K. K. Hood, D. M. Maahs and K. S. Burger, *Diabet Med*, 2017, **34**, 1500-1507.
9. D. Elleri, D. B. Dunger and R. Hovorka, *BMC Med*, 2011, **9**, 120.
10. V. Foerster and M. Severn, in *CADTH Issues in Emerging Health Technologies*, Ottawa (ON), 2016, pp. 1-11.
11. H. Thabit and R. Hovorka, *Endocrinol Metab Clin North Am*, 2012, **41**, 105-117.
12. H. Thabit and R. Hovorka, *Curr Opin Endocrinol Diabetes Obes*, 2014, **21**, 95-101.

13. T. G. Farmer, Jr., T. F. Edgar and N. A. Peppas, *J Pharm Pharmacol*, 2008, **60**, 1-13.
14. L. Zhao, Q. Huang, Y. Liu, Q. Wang, L. Wang, S. Xiao, F. Bi and J. Ding, *Materials (Basel)*, 2017, **10**, 170.
15. S. Ferri, K. Kojima and K. Sode, *J Diabetes Sci Technol*, 2011, **5**, 1068-1076.
16. E. H. Yoo and S. Y. Lee, *Sensors (Basel)*, 2010, **10**, 4558-4576.
17. H. C. Wang and A. R. Lee, *J Food Drug Anal*, 2015, **23**, 191-200.
18. T. Traitel, Y. Cohen and J. Kost, *Biomaterials*, 2000, **21**, 1679-1687.
19. S. I. Kang and Y. H. Bae, *J Control Release*, 2003, **86**, 115-121.
20. X. Qi, W. Wei, J. Li, G. Zuo, X. Pan, T. Su, J. Zhang and W. Dong, *Mol Pharm*, 2017, **14**, 431-440.
21. W. Zhao, H. Zhang, Q. He, Y. Li, J. Gu, L. Li, H. Li and J. Shi, *Chem Commun (Camb)*, 2011, **47**, 9459-9461.
22. L. Zhao, C. Xiao, L. Wang, G. Gai and J. Ding, *Chem Commun (Camb)*, 2016, **52**, 7633-7652.
23. L. Zhao, L. Wang, Y. Zhang, S. Xiao, F. Bi, J. Zhao, G. Gai and J. Ding, *Polymers (Basel)*, 2017, **9**.
24. J. Yu, Y. Zhang, Y. Ye, R. DiSanto, W. Sun, D. Ranson, F. S. Ligler, J. B. Buse and Z. Gu, *Proc Natl Acad Sci U S A*, 2015, **112**, 8260-8265.
25. J. Yu, Y. Zhang and Z. Gu, *Methods Mol Biol*, 2017, **1570**, 251-259.
26. J. Cegla, *Ann Clin Biochem*, 2015, **52**, 720.
27. W. Tai, R. Mo, J. Di, V. Subramanian, X. Gu, J. B. Buse and Z. Gu, *Biomacromolecules*, 2014, **15**, 3495-3502.
28. W. Qi, X. Yan, J. Fei, A. Wang, Y. Cui and J. Li, *Biomaterials*, 2009, **30**, 2799-2806.
29. K. Podual, *Polymer*, 2000, **41**, 3975-3983.
30. X. Li, M. Fu, J. Wu, C. Zhang, X. Deng, A. Dhinakar, W. Huang, H. Qian and L. Ge, *Acta Biomater*, 2017, **51**, 294-303.
31. Z. Gu, T. T. Dang, M. Ma, B. C. Tang, H. Cheng, S. Jiang, Y. Dong, Y. Zhang and D. G. Anderson, *ACS Nano*, 2013, **7**, 6758-6766.

32. S. B. Bankar, M. V. Bule, R. S. Singhal and L. Ananthanarayan, *Biotechnol Adv*, 2009, **27**, 489-501.
33. M. J. Webber and D. G. Anderson, *J Drug Target*, 2015, **23**, 651-655.
34. W. C. Blocher and S. L. Perry, *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, 2017, **9**.
35. H. Chu, J. Gao, C. W. Chen, J. Huard and Y. Wang, *Proc Natl Acad Sci U S A*, 2011, **108**, 13444-13449.
36. K. A. Black, D. Priftis, S. L. Perry, J. Yip, W. Y. Byun and M. Tirrell, *ACS Macro Letters*, 2014, **3**, 1088-1091.
37. N. Martin, M. Li and S. Mann, *Langmuir*, 2016, **32**, 5881-5889.
38. N. Pippa, M. Karayianni, S. Pispas and C. Demetzos, *Int J Pharm*, 2015, **491**, 136-143.

Chapter 6

Magnetically Responsive Peptide Coacervates with Dual Triggered Hyperthermia and Chemotherapy for Liver Cancer Treatment

Liver cancer is a malignancy linked to high rates of morbidity and mortality. While chemotherapeutic drugs like Dox can be used to delay the liver cancer progression, its long-term effectiveness is limited and routine usage of Dox at effective concentrations is hampered by its severe side effects. Here, Dox and MNPs are first encapsulated within DgHBP-2 peptide coacervates. These Dox-loaded magnetic coacervates (DMCs) are used for thermo-chemotherapy in HepG2 liver cancer cells, where an external AMF can be used to control the release of Dox. Application of external AMF on DMCs produce a temperature of 45°C, resulting in the disassembly and subsequent release of Dox. This release of Dox and heat generated from DMCs allows dual chemotherapy and hyperthermia for a more effective cancer therapy. In vitro studies have shown that DMCs have low toxicity and are greatly effective in inducing HepG2 cell death under AMF. Thermo-chemotherapy treatment on HepG2 cells have also been shown to be more effective when compared to either chemotherapy or hyperthermia treatments alone. In summary, these results demonstrate that DMCs can be useful in future theragnostic strategies in liver cancer by offering wireless and programmable control of Dox release.

The chapter is under revision as Zhi Wei Lim, Vijaykumar B. Varma, Raju V. Ramanujan, Ali Miserez, *Magnetically Responsive Peptide Coacervates with Dual Triggered Hyperthermia and Chemotherapy for Liver Cancer Treatment*, Acta biomaterialia.

6.1 Introduction

Liver cancer is an aggressive cancer related to high mortality worldwide and remains an extremely difficult cancer to treat.^{1, 2} Although conventional cancer treatments, *e.g.* surgical and chemotherapy are useful in treatment of early stage liver cancer, these treatments are frequently ineffective against late stage liver cancer.¹⁻³ Traditional surgeries can be useful in removing localised tumours, however they are not able to avert cancer metastasis. Alternatively, chemotherapy uses cytotoxic drugs that flow into the whole body through blood circulation. Yet, liver cancer are generally chemotherapy refractory and limited chemotherapeutic drugs such as 5-fluorouracil and Doxorubicin are effective.⁴⁻⁶ These chemotherapeutic drugs are often unspecific, leading to serious unintentional and undesirable side effects like anaemia and bleeding.^{7, 8} These serious side effects also contribute to the high mortality rates seen in many cancer patients. Furthermore, penetration of chemotherapeutic drugs into tumour tissues are poor, leading to the use of higher drug concentration, ultimately resulting in higher toxicity and drug resistance.

The application of magnetic field in targeted drug delivery was first seen in 1980s and has been increasingly popular in the last decade⁹, due to the production of stronger permanent magnets and cutting-edge magnetic probes, allowing the combination of diagnostics and therapeutics for better treatment efficiency. Unlike infrared and visible light, magnetic fields are absorbed poorly and hence can penetrate deeper into the tissues.¹⁰ Besides targeted delivery, localised heating can be induced by magnetic fields when electromagnetic energy within MNPs is converted into thermal energy.⁹⁻¹³ Localised heating, also known as MHT, has been used in thermo-chemotherapy, for triggering drug release and for inducing cancer cell death.¹⁴⁻¹⁶ In fact, MNPs have been frequently used as theranostic agents due to its ability to merge MHT and magnetic resonance imaging into a single cancer treatment.^{13, 17-20} Many previous studies have also revealed that thermo-chemotherapy is more effective at inducing cancer cell death and improving the survival rate of patients when compared to chemotherapy or hyperthermia treatments alone.^{12, 21-23} Therefore, substantial research has been dedicated to the fabrication of chemotherapeutic drug-loaded MNPs and heat-sensitive drug delivery systems.^{11, 13, 24-30}

While there are many different types of carriers that have been used in “smart” delivery systems, coacervates demonstrate specific advantages such as high biocompatibility, fast self-assembly in an aqueous environment, shear-thinning and ultra-low surface tension (convenient for delivery and intravenous injection), free of organic solvents (retention of protein drug efficacy) and high loading capacity.³¹⁻³⁷ One important characteristic of coacervates is their ability to rapidly package and encapsulate a huge range of therapeutic molecules during the coacervation process, including hydrophilic and hydrophobic compounds, high and low-molecular weight molecules or other biomacromolecules, thus opening exciting opportunities for drug delivery applications.³⁶⁻³⁹ Even so, coacervates do not have in-built targeted delivery and controlled release properties, which are essential in “smart” drug delivery systems. In addition, well-known coacervation systems based on elastin are not suitable for heat-responsive drug delivery systems due to their lower-critical solution temperature (LCST),^{40, 41} by which heating stimulates coacervation instead of dissociation that is needed for release of drug.⁴²

In this chapter, DMCs were fabricated for thermo-chemotherapy, allowing wireless with programmable magnetic control for navigated delivery and controlled release of Dox (Scheme 6.1). Dox was selected to be the model chemotherapeutic drug due to its effectiveness against liver cancer and its autofluorescence that allows easy visualisation. DMCs were prepared by co-encapsulate of Dox and MNPs with DgHBP-2 peptide, followed by crosslinking to stabilise these micro-droplets.

Figure 6.1. Illustration of DMCs for thermo-chemotherapy. (A) DMCs are formed by encapsulation of Dox and MNPs into DgHBP-2 peptide coacervates. Dox and MNPs are first pipetted into phosphate buffer at their respective concentrations, DgHBP-2 peptide solution is next added to induce coacervation and encapsulation. (B) When DMCs reach the tumour site, MHT produced by MNPs under AMF triggers heat-induced disassembly of DMCs and ultimately release of Dox.

6.2 Experimental methods

6.2.1 Materials

Dox was bought from twcbio Pte Ltd. Ferrofluid (EMG 707 (resuspended in water), Ferrotec, Singapore) of 2% v/v MNPs concentration, saturation magnetization: 11 mT, initial magnetic susceptibility: 1.51, viscosity: 1.78 ± 0.02 mPa·s, and density: 1.110 ± 0.002 g cm⁻³ was used for magnetic studies. 4-methylcatechol, amiloride hydrochloride, chloroquine diphosphate crystalline cytochalasin B, chlorpromazine hydrochloride, methyl- β -cyclodextrin, sodium azide (NaN₃) and sodium periodate (NaIO₄) were bought from Sigma Aldrich (USA). HepG2 liver cancer cells and NIH3T3 mouse fibroblast were cultured at 37°C in 5% CO₂.

6.2.2 Encapsulation of Dox and MNPs by coacervates

Dox (dissolved in water, final concentration is 0.04 mg/mL) and MNPs (dissolved in water, final concentration is 0.008% v/v) were first pipetted into the phosphate buffer (0.1 M, pH 9.5) to their final concentrations. DgHBP-2 peptide (dissolved in 10 mM acetic acid, stock solution: 10 mg/mL) was next added to the reaction mixture to a 1:9 ratio to initiate encapsulation and coacervation. Mild pipetting was done to mix the peptide and Dox/MNP mixture evenly.

6.2.3 Fluorescence and optical microscopy

The experimental method used in this section was same as in chapter 5.2.4.

6.2.4 Magnetic alignment of coacervates

Alignment of coacervates was done in a uniform magnetic field that was generated by two facing permanent magnets (Nd-Fe-B, Dimension: 25 mm (L) × 12.5 mm (W) × 12.5 mm (H)). The strength of the field and its distribution was analysed along $\pm x$ and z axis using a MG-4D gaussmeter (Walker scientific Inc, Australia). The uniform magnetic field setup and its distribution were revealed in Figure 6.2. A magnetic field (magnetic strength: ~200 mT) was applied during the magnetic alignment of DMCs.

Figure 6.2. Magnetic alignment of DMCs under uniform magnetic field. (A) illustration of the experimental setup. (B) Magnetic strength and field distribution of the two permanent magnets on the $\pm Y$ and $+Z$ directions.

6.2.5 Encapsulation efficiency

The experimental method used in this section was same as in chapter 5.2.6 except that excitation and emission wavelength used is 470 nm and 590 nm respectively.

6.2.6 Concentration of Dox within coacervates

Dox-loaded coacervates were first added to a glass slide and focused using an upright fluorescence microscope (Nikon Eclipse Ci) attached with a spectrophotometer (Avantes avaspec-ULS2048 Spectrometer) and a camera (Nikon DS-Ri2). Unencapsulated Dox was subsequently washed off the slide using phosphate buffer. An area of $5\ \mu\text{m}$ by $5\ \mu\text{m}$ square (probe area) was used to analyse the fluorescence of Dox within individual coacervate. The fluorescence of unencapsulated Dox (0.04 mg/mL to 0.2mg/mL) was measured with same probe area to generate a standard curve. The concentration of Dox within individual coacervates was then back-calculated using the standard curve. Micrographs were exported using NIS-elements (Nikon) and Dox fluorescence were analysed by Avasoft 8.0 (Avantes).

6.2.7 Crosslinking of DgHBP-2 peptide coacervates

The experimental method used in this section was same as in chapter 4.2.9.

6.2.8 Dynamic light scattering (DLS)

The experimental method used in this section was same as in chapter 4.2.3

6.2.9 MTT assay

The experimental method used in this section was same as in chapter 4.2.13

6.2.10 Live/dead staining

The experimental method used in this section was same as in chapter 4.2.14

6.2.11 Scanning Electron microscopy

The experimental method used in this section was same as in chapter 4.2.10

6.2.12 Transmission Electron Microscopy

The experimental method used in this section was same as in chapter 4.2.11

6.2.13 Fluorescence recovery after photobleaching (FRAP)

FRAP experiments were done on the LSM 710 confocal microscope (Carl Zeiss) fitted with a 63× oil objective lens and FRAP wizard software. Photobleaching of DMCs was achieved with a 488 nm laser, and the bleaching was stopped once the fluorescence intensity of the region of interests (ROIs) was decreased to 40% of the initial intensity. Mean fluorescence intensities for the ROIs were measured using ImageJ and plotted in Origin 9.0 software.

6.2.14 LysoTracker staining

HepG2 cells was seeded at concentration of 5×10^4 cells/mL into 4-well Lab-Tek chambered glass slide (Thermofisher Scientific) and incubated for 24 hours at 37°C. Next, HepG2 cells were washed 2 times using PBS. Cell culture media containing GFP-loaded coacervates were subsequently added to the cells and incubated for another 24 hours. After washing, cell culture media with LysoTracker Red DND-99 (5 μ M, Life Technologies) were added to the cells and incubated for another hour. The cells were subsequently washed

and then fixed with 4% paraformaldehyde (PFA) at room temperature for 20 min. After washing, the cells were stained with Hoechst 33342 (2 µg/mL, Sigma Aldrich) for 15 min, followed by wheat germ agglutinin (WGA) conjugated to Alexa Fluor 633 (1 µg/mL, ThermoFisher Scientific, USA) for 10 mins in PBS at room temperature. The cells were imaged on a laser confocal scanning microscope (True Confocal Scanner SP8, Leica) using the following wavelengths: Hoechst 33342 (Excitation: 346 nm, Emission: 460 nm), eGFP (Excitation: 448 nm, Emission: 509 nm), LysoTracker red DND 99 (Excitation: 577 nm, Emission: 590 nm), and WGA-633 Alexa Fluor (Excitation: 632 nm, Emission: 647 nm). Micrographs were post-processed and exported using LAS X software.

6.2.15 Uptake assay with endocytosis inhibitors

HepG2 cells were seeded at a density of 8×10^4 cells/mL into 4-well Lab-Tek chambered glass slide (ThermoFisher Scientific, USA) and incubated for 24 hours at 37°C. Chloroquine and chlorpromazine are inhibitors of clathrin-mediated endocytosis; amiloride hydrochloride is inhibitor of macropinocytosis; methyl-β-cyclodextrin is inhibitor of caveolae-mediated endocytosis; cytochalasin B and NaN₃ are inhibitors of micropinocytosis and ATP-dependent endocytosis respectively. HepG2 cells were incubated with these inhibitors for an hour before GFP-loaded coacervates were added. The cells were washed with PBS after 4 hours of incubation, and subsequently fixed with 4% PFA, followed by staining with Hoechst 33342 (2 µg/mL) and WGA-Alexa Fluor 633 (1 µg/mL). Micrographs were taken using confocal microscopy (True Confocal Scanner SP8, Leica).

6.2.16 *In vitro* release assay (Temperature triggered Dox release)

50 µL of 4-MC/NaIO₄ crosslinked DMCs were diluted to 200 µL with 1X PBS and placed in a 2.0 mL microcentrifuge tube (Eppendorf, Germany). The coacervates were next incubated in water baths of respective temperatures (25°C to 45°C) for 48 hours. At each timepoint, the coacervates were centrifuged (4000 rpm, 5min) and the fluorescence of Dox within the supernatant was measured with a Fluorolog fluorescence spectrophotometer (HORIBA Jobin Yvon, Japan, Excitation: 470 nm and Emission: 550 nm). The removed supernatant was substituted with new PBS at each timepoint.

6.2.17 Temperature measurement of coacervates under AMF

4-MC/NaIO₄ Crosslinked DMCs were placed within a microcentrifuge tube (Eppendorf, Germany) and positioned within the copper coils of AMF generator (Inductelec Limited, United Kingdom) with these power settings (4 kA/m, 375 kHz). Realtime temperature measurement were done using a M600 fiber optic temperature sensor (Luxtron, Dubai).

6.2.18 Cumulative release of Dox over time

4-MC/NaIO₄ crosslinked DMCs were placed in AMF generator (Inductelec Limited, United Kingdom) as described in section 6.2.15. 100 µL of coacervates were removed from the AMF generator at different timings, followed by centrifugation (4000 rpm, 5 min) and measurement of Dox fluorescence (Excitation: 470 nm and Emission: 550 nm).

6.2.19 Cumulative release of Dox by multiple AMF cycles

4-MC/NaIO₄ crosslinked DMCs were subjected to multiple AMF cycles, with each cycle consisted of 20 min heating followed by 10 mins of resting. After each cycle, 100 µL of the coacervates were pipetted out and its supernatant was analysed for measurement of Dox fluorescence (Excitation: 470 nm and Emission: 550 nm).

6.2.20 Specific absorption rate (SAR)

SAR of ferrofluids is calculated using the below equation^{14, 16}:

$$SAR = C \left(\frac{\Delta T}{\Delta t} \right) mass(ferrofluids)/mass(MNPs)$$

Where, C is heat capacity of ferrofluids and ($\Delta T/\Delta t$) is the gradient of temperature against time graph.

6.2.21 Intrinsic loss of power (ILP)

ILP (nH·m²/kg) of ferrofluids is calculated using the below equation⁴³:

$$ILP = SAR/(H^2 f)$$

Where, H (kA/m) and f (kHz) represent the applied magnetic field strength and the used frequency, respectively.

6.3.1 Encapsulation of Dox by DgHBP-2 peptide coacervates

Dox was loaded into DgHBP-2 coacervates at different buffers to determine the optimum pH for encapsulation of Dox. Co-localisation of Dox was confirmed by the red fluorescence within the coacervates micro-droplets. Of the three tested pH (pH 7.5, pH 8.5, and pH 9.5), the optimum pH condition for Dox encapsulation was found to be pH 9.5 (Figure 6.3). This is due to the highly positively charged nature of Dox (pKa: 8.2) at physiological pH, resulting in charge-charge repulsion. Since Dox becomes uncharged at pH 8.2 to 9.5, its encapsulation was more efficient in pH 9.5 buffer.

Figure 6.3. Encapsulation of Dox by DgHBP-2 peptide coacervates in different pH buffers. Left: light micrographs; Middle: fluorescence micrographs; Right: merged channels micrographs.

Without magnetic field, DMCs were randomly deposited onto the glass coverslip (Figure 6.4A). When a uniform magnetic field (200 mT) was applied, the DMCs rapidly

aligned into chains of DMCs (Figure 6.4B), signifying that DgHBP-2 peptide could encapsulate Dox and MNPs concurrently and were magnetically responsive.

Figure 6.4. Micrographs of DMCs. (A) without magnetic field and (B) with magnetic field.

From table 6.1, the EE% of Dox by DgHBP-2 peptide coacervates was around ~40%. Since coacervates are known have high loading capacity and can amass high concentrations of macromolecules,^{44, 45} the concentration of Dox inside each coacervate was studied. The concentration of Dox inside each coacervates was found to be ~0.12 mg/mL, which was ~300% higher than the original Dox concentration within the phosphate buffer (table 6.2).

Table 6.1. EE% of different concentrations of Dox by DgHBP-2 peptide coacervates. ($n = 3$, mean $\pm$ S.D.)

Doxorubicin ($\mu\text{g/mL}$)	DgHBP-2 peptide (mg/mL)	EE (%)
1	1.0	3.6 ± 3.3
3	1.0	16.5 ± 3.7
5	1.0	23.0 ± 1.2
10	1.0	31.6 ± 2.4
40	1.0	38.2 ± 2.6

Table 6.2. Concentration of Dox within DgHBP-2 peptide coacervates. ($n = 3$, mean $\pm$ S.D.)

Dox-loaded coacervates	Fluorescence (arbitrary unit)	Concentration (mg/mL)
1	1608	0.13
2	1432	0.11
3	1716	0.13
4	1449	0.11
5	1716	0.13
6	1746	0.14
7	1470	0.11
8	1485	0.12
9	1395	0.11
10	1384	0.11
Average: 0.12		
Standard deviation: 0.01		
Fold change = (Dox concentration within coacervate) / (Initial Dox concentration) $\times$ 100 (%)		$\frac{0.12}{0.04} \times 100 = 300\%$

6.3.2 Crosslinking of DMCs by different methods

Following the recruitment of Dox within the coacervate micro-droplets, excess of Dox remaining in solution has to be removed by successive washing and centrifugation steps. However, during centrifugation, the microdroplets stuck to the side of the Eppendorf tubes and could not be recovered. Therefore, the coacervates were cross-linked in order to stabilize them and prevent adhesion to the test tubes. DMCs were crosslinked using different methods developed in chapter 4. With this cross-linking step, excess of Dox in solution can now be wash off and recover the micro-droplets. First, DMCs were prepared using DgHBP-2 peptide DOPA and then crosslinked using FeCl_3 or NaIO_4 . The red fluorescence of Dox in DOPA- FeCl_3 crosslinked coacervates were less intense when compared to the un-crosslinked coacervates, indicating that Dox was not retained within

the coacervates after crosslinking and washing procedures (Figure 6.5A and B). Hence DOPA-FeCl₃ coordination crosslinking was not suitable for the preparation of crosslinked DMCs. In contrast to DOPA-FeCl₃ crosslinked DMCs, the red fluorescence of Dox within DOPA- NaIO₄ crosslinked DMCs were similar to that of un-crosslinked coacervates. Although many of the DOPA- NaIO₄ crosslinked DMCs were aggregating together (Figure 6.5C), they remained magnetically responsive (Figure 6.5D). However, DOPA- NaIO₄ crosslinked DMCs were found to be extremely stable and heating does not trigger the release of Dox, making these DMCs not usable in heat-responsive drug delivery systems.

Next, DMCs was prepared with DgHBP-2 peptide tyrosine and crosslinked using 4-MC and NaIO₄. The catechol moieties on the 4-MC are rapidly oxidised into 4-Methyl-1,2-benzoquinone (4-MB) by using a chemical oxidant e.g. NaIO₄.⁴⁶ The resulting 4-MB is an o-quinone, that is electrophilic and particularly reactive with neutrophilic groups like histidine's imidazoles and amines.⁴⁶ Dox remained within the coacervates after crosslinking procedures (Figure 6.6A) and were magnetically responsive under uniform magnetic field (Figure 6.6B). The surface morphology of DMCs in SEM micrographs had shown that it is similar to crosslinked coacervates, demonstrating that MNPs were inside and not just attached onto the peripheral of crosslinked coacervates (Figure 4.20A to B and 6.7). TEM micrographs had also confirmed that the presence of MNPs within the DMCs (Figure 6.8, brown arrows). "Budding" effect was also similarly seen in DMCs, indicating that the surface of the crosslinked coacervates was dynamic (Figure 6.8, purple arrows). To verify the liquid-like properties of cross-linked DMCs, fluorescence recovery after photobleaching (FRAP) experiments was done to establish the dynamic nature of crosslinked DMCs. ~80% recovery of fluorescence was observed after 60 seconds, indicating that DMCs remain dynamic even after crosslinking (Figure 6.09).

Figure 6.5. Fluorescence micrograph of crosslinked DMCs by different methods. (A) un-crosslinked DMCs, **(B)** DOPA-FeCl₃ crosslinked DMCs, DOPA-NaIO₄ crosslinked DMCs **(C)** without magnetic field and **(D)** with magnetic field.

Figure 6.6. Fluorescence micrographs of 4-MC/NaIO₄ crosslinked DMCs. (A) without magnetic field and **(B)** with magnetic field.

Figure 6.7. SEM micrographs of 4-MC/NaIO₄ crosslinked DMCs. (A) low magnification field and (B) higher magnification

Figure 6.8. TEM micrographs of 4-MC/NaIO₄ crosslinked DMCs. (A) low magnification field and (B) higher magnification. Red arrows and brown arrows represent DMCs and MNPs respectively; purple arrows indicate the budding effect seen on top of DMCs. The webbed-like net structures (green arrows) seen under the coacervates in the micrographs are the lacey carbon support film that are part of the TEM copper grids.

Figure 6.9. Quantification of fluorescence signal from crosslinked DMCs in FRAP experiments ($n = 6$, $mean \pm S.D.$). Fluorescence recovery of $\sim 80\%$ was reached in DMCs 60s after photobleaching.

DLS detected two populations of DMCs (centered at *ca.* 650 nm and 3 μm) before crosslinking (Figure 6.10A), which is attributed to the liquid-like nature of coacervates that can result in Ostwald ripening or fusion of individual droplets. After crosslinking, the size of these two populations of DMCs decreased slightly to *ca.* 550 nm and 2.2 μm and remained stable for at least 15 days in PBS buffer (Figure 6.10B to 6.10F). The amount of Dox within DMCs after crosslinking was found to be comparable to the amount of Dox obtained from the encapsulation efficiency assays (Figure 6.11), indicating that Dox remains within the micro-droplets following the crosslinking and washing steps. Furthermore, red fluorescence was detected in micrographs of crosslinked DMCs for at least 15 days, demonstrating long term stability of the DMCs while retaining their Dox cargo (Figure 6.12).

Figure 6.10. DLS of DMCs (A) before and (B-F) after crosslinking in PBS. Two distribution size of DMCs were observed before and after crosslinking, which were similar and remained stable for at least 15 days. ($n = 5$)

Figure 6.11. Fluorescence intensity of Doxorubicin (calculated concentration of Dox within DMCs) and crosslinked DMCs. Minimal loss of Dox was observed from the DMCs after crosslinking. ($n = 3$, mean $\pm$ S.D.)

Figure 6.12. Fluorescence micrographs of DMCs (A) before crosslinking and (B-F) after crosslinking in PBS. Crosslinked DMCs are stable in PBS for at least 15 days without significant loss of the Dox cargo (red fluorescence).

6.3.3 Cellular uptake of coacervates

The cellular uptake and intracellular distribution of coacervates were next studied using HepG2 liver cancer cells. HepG2 cells were treated with 100 $\mu\text{g/mL}$ of green fluorescence protein (GFP)-loaded coacervates for 24 hours, followed by endosomal (lysotracker probes) staining and observation under confocal microscope. GFP-loaded coacervates with and without crosslinking were uptake efficiently into HepG2 cells. Strong green fluorescence from GFP-loaded coacervates was observed as puncta-like staining within the cytoplasm surrounding the nucleus membrane, but not inside nucleus (Figure 6.13). From Figure 6.14, the green fluorescence of GFP (green arrows) does not co-localised with red fluorescence of lysotracker probes (red arrows), signifying that either the coacervates had already escaped out of endosomes after uptake or endocytosis was not the dominant pathway for coacervates uptake.

Figure 6.13. Confocal micrographs (3D view) of HepG2 cells treated with GFP-loaded coacervates and lysotracker probes. (A, B) untreated cells. (C, D) un-crosslinked coacervates. (E, F) 4-MC/NaIO₄ crosslinked coacervates. Green fluorescence (GFP-loaded coacervates) was observed beneath the cell membrane (white box), indicating uptake of coacervates into the cells. Red and white fluorescence represents endosomes and cell membranes.

Figure 6.14. Confocal micrographs of HepG2 cells treated with GFP-loaded coacervates (100 $\mu\text{g/mL}$) and lysotracker probes. (A, D) Control cells. (B, E) GFP loaded un-crosslinked coacervates. (C, F) GFP loaded 4-MC/NaIO₄ crosslinked coacervates. **Top rows: z-stack merged images. **Bottom rows:** z-stack merged images with membrane staining. GFP-loaded coacervates (green fluorescence, green arrow) and endosomes (red fluorescence, red arrow) were not co-localized. White fluorescence represents the cell membrane.**

To test these two hypotheses, control uptake experiments were performed in the presence of different endocytosis inhibitors. Since the most well-known endocytosis pathway for uptake of macromolecules is clathrin-mediated endocytosis (CME),⁴⁷ the effects of CME inhibitors (chloroquine and chlorpromazine) was first investigated.^{48, 49} Confocal micrographs of HepG2 cells (pre-treated with CME inhibitors) had shown efficient uptake of crosslinked coacervates into the cytoplasm, signifying that chlorpromazine and chloroquine have minimal effect on the cellular uptake of crosslinked coacervates (Figure 6.15A and B). These data suggest that coacervates uptake was not dependent on CME pathway. Next, the effects of pinocytosis inhibitors (amiloride hydrochloride and cytochalasin B) on uptake of coacervates into HepG2 cells were tested.^{48,}

⁴⁹ As shown in Figure 6.15C and D, cellular uptake of crosslinked coacervates into HepG2 cells was not inhibited by these pinocytosis inhibitors. Likewise, caveolin-mediated endocytosis inhibitor (methyl- β -cyclodextrin) did not prevent the cellular uptake of crosslinked coacervates (Figure 6.15E).⁴⁹ Lastly, the effect of ATP-dependent endocytosis inhibitor, sodium azide (NaN_3) was examined.⁴⁸ From Figure 6.15F, NaN_3 also did not have a substantial effect on cellular uptake of coacervates. Although the actual mechanism of coacervates uptake into cells is still unknown, these data strongly suggest that the uptake mechanism is not dependent on ATP or endocytosis pathway. Besides using chemical endocytosis inhibitors, cellular uptake of coacervates were also tested at 4°C (Figure 6.16). At 4°C, the cellular uptake of un-crosslinked coacervates was greatly reduced (Figure 6.16A) whereas the uptake of crosslinked coacervates was inhibited (Figure 6.16B). The puncta-like staining of coacervates was no longer seen in the treated cells. At 4°C, not only the active uptake is inhibited, the rigidity of the plasma membrane also increases, resulting in lesser passive uptake.^{50,51} Together with the results obtained in Figure 6.15F, (uptake in NaN_3 treated cells was not inhibited), these data suggest that the rigidity of plasma membrane has an important role in the uptake of coacervates.

6.3.4 *In vitro* heat triggered release of Dox

Next, the release of Dox from the coacervates micro-droplets after heat treatment was explored. 4-MC/ NaIO_4 crosslinked DMCs were first incubated at temperatures between 25°C to 45°C, followed by quantification of Dox fluorescence within the supernatant. This range of temperature were carefully chosen to match the temperatures that would eventually attain using AC magnetic field. From figure 6.17, some leakage of Dox from DMCs at physiological temperature (37°C) and below (25°C) was observed, however the amount of Dox release was much higher and with a faster rate at 42°C and 45°C, demonstrating that DMCs permit for a more prominent Dox release at temperatures above physiological temperature.

Figure 6.15. Confocal micrographs of HepG2 cells treated with endocytosis inhibitors (orthographic view). (A, B) CME inhibitors chlorpromazine (30 μM) (A) and chloroquine (100 μM) (B). (C, D) Pinocytosis inhibitors amiloride hydrochloride (20 μM) (C) and cytochalasin B (8.5 μM) (D). (E) Methyl- β -cyclodextrin (1 mM), and (F) NaN_3 (10 mM). Green fluorescence and white colour represent GFP-loaded coacervates and the cell membrane, respectively.

Figure 6.16. Cellular uptake of GFP loaded coacervates by HepG2 cells at 4°C (orthographic view). (A) un-crosslinked coacervates. (B) 4-MC/NaIO₄ crosslinked coacervates. Green fluorescence and white colour represent GFP-loaded coacervates and the cell membrane, respectively. Cells were first incubated at 4°C for 30 mins before treated with GFP-loaded coacervates. Confocal imaging was done after 2 hours of incubation with coacervates at 4°C.

Figure 6.17. Release of Dox from 4-MC/NaIO₄ crosslinked DMCs (100 μg/mL) at different temperatures. (*n* = 3, mean ± S.D.)

6.3.5 Cytotoxicity efficacy of released Dox from DMCs

In order to examine the cytotoxicity of released Dox, the DMCs were first heated at 45°C for different durations and subsequently incubated with HepG2 cells (Figure 6.18). Pre-heated crosslinked DMCs induced significantly more HepG2 cell death when compared to non-preheated DMCs, signifying that in the absence of heat treatment, Dox leakage from DMCs at physiological temperature was inadequate to induce HepG2 cell death (Figure 6.18A, cell viability was ~90%). When the length of pre-heating increases from 30 min to 6 hours, increasing amount of Dox was release from DMCs, resulting in more cell death (Figure 6.18E to H). These data were further confirmed by the results from MTT assay where the relative cell viability of DMCs treated HepG2 cells decreased with increasing length of DMCs pre-heating (Fig. 6.19B to D, cell viability decreased to ~20%).

Figure 6.18. Live/dead staining of 4-MC/NaIO₄ crosslinked DMCs treated HepG2 cells. (A) Control cells (without treatment). (B) Control dead cells (without treatment, fixed with 70% methanol). (C) Crosslinked coacervates (without Dox and MNPs, 100 μg/mL). (D-H) crosslinked DMCs (100 μg/mL) without pre-heating (D); 30 min of pre-heating (F); 1 hour of pre-heating (G); 2 hours of pre-heating (G); and 6 hours of pre-heating (H).

Figure 6.19. Relative cell viability of pre-heated 4-MC/NaIO₄ crosslinked DMCs treated HepG2 cells. (A) no pre-heating. (B) 30 min of pre-heating. (C) 2 hours of pre-heating. (D) 6 hours of pre-heating. ($n = 6$, mean $\pm$ S.D.)

6.3.6 Magnetic studies of crosslinked DMCs for thermo-chemotherapy

Superparamagnetic nanoparticles generate heat under AMF, a phenomenon generally known as MHT.⁵²⁻⁵⁵ Heat generation is a result of Néel relaxation (rotation of magnetic moment) and/or Brownian relaxation (rotation of MNPs). The ferrofluids used in these experiments exhibit a SAR value and intrinsic loss power of 100.6 W/g and 16.67 nHm²/kg, respectively, calculated from the gradient of the ferrofluid heating curve (Figure 6.20). To measure the amount of heat release from 4-MC/NaIO₄ crosslinked DMCs, the temperature of these DMCs were measured over 40 min of AMF treatment (magnetic field strength: 4 kA/m and frequency: 375 kHz). From Figure 6.21A, 4-MC/NaIO₄ crosslinked DMCs achieved over ~50°C with 40 min of AMF treatment. In the absence of crosslinked DMCs or MNPs, a temperature of ~38°C was achieved under identical AMF conditions. This slight increase in temperature is similar to physiological body temperature (37°C) and is due to heat produced by AMF equipment.

Figure 6.20. The heating curve of Ferrofluid and DMCs (under AMF) used in SAR calculation.

Figure 6.21. Magnetic studies of 4-MC/NaIO₄ crosslinked DMCs under AMF (A) Temperature of crosslinked DMCs over 40 mins of AMF. MNPs diluted in PBS act as positive control. (B) Cumulative release of Dox vs time during a single cycle of AMF, and at 25 °C and 37 °C in the absence of AMF. (C) Cumulative release of Dox with multiple AMF cycles (Each cycle: 20 min of AMF with 10 mins of cooling), equivalent to a local temperature of ca. 45°C. (D) Pulsatile release of Dox from DMCs by several cycles of AMF, heating the DMCs to 45°C, then cooling to room temperature (28°C). Pink and purple columns represent AMF “ON” and “OFF” respectively. Concentration of DMCs and MNPs were 500 µg/mL and 0.04% v/v, correspondingly. ($n = 3$, mean $\pm$ S.D.)

To determine if Dox release from DMCs could be triggered by AMF, 4-MC/NaIO₄ crosslinked DMCs (resuspended in PBS) were prepared and exposed to AMF for different length of time. The amount of Dox being released from DMCs slowly increased with increasing time of AMF treatment. ~25% of Dox was released from DMCs during the 40 min of AMF (as compared to less than 5% at 25 °C and 37 °C in the absence of AMF),

indicating controlled release of Dox by MHT can be achieved (Figure 6.21B). This modest amount of Dox released from DMCs with one AMF cycle has useful implications because: (i) DMCs could act as a Dox reserve and any additional Dox that are needed can be released by undergoing more AMF cycles; (ii) slow release of Dox can prevent healthy cells from being damaged during cancer treatment.; and (iii) a single cycle of AMF cycle triggered the release of ~25% Dox. Hence, the amount of Dox release from DMCs can be adjusted between 5% and 25% by changing the length of AMF. To confirm this hypothesis, 4-MC/NaIO₄ crosslinked DMCs were subjected to consecutive AMF cycles and then observed for pulsatile release of Dox. Each AMF cycle consists of 20 min of AMF with the purpose of allowing the DMCs to reach a temperature of ~45°C (Figure 6.21A, temperature required for hyperthermia treatment), followed by 10 mins of cooling. As shown in Figure 6.21C, incremental amounts of Dox were released from DMCs with the additional AMF cycles and this release could be repeated for at least 5 cycles. Subsequently, the AMF “ON” and “OFF” responsiveness of DMCs was examined. DMCs were first heated to 45°C using AMF and then cooled down to room temperature (28°C). Dox was released from DMCs only under AMF-induced heating, while the release of Dox was diminished after cooling for at least 5 successive heating/cooling cycles (Figure 6.21D). These data show that only a portion of Dox was released with each AMF cycle, in agreement with the hypothesis that DMCs could act as a Dox reservoir and signifying repeatable controlled release of precise amount of Dox under AMF. The size distribution and fluorescence intensity of DMCs were also examined after multiple cycles of AMFs (Figure 6.22). Both the size of DMCs as well as their fluorescence intensity decreased with increasing the number of AMF cycles, with the lower (sub-micron) size population almost disappearing after a few cycles (Figure 6.22E, 6.22G and 6.22I). These results substantiate that DMCs underwent progressive dissociation and that the Dox cargo was concomitantly released upon exposure to AMF cycles.

Figure 6.22. DLS and micrographs of crosslinked DMCs after AMF treatment. (A and B) 1× AMF cycle; (C and D) 2× AMF cycle; (E and F) 3× AMF cycle; (G and H) 4× AMF cycle and (I and J) 5× AMF cycle. Each AMF cycle was 20 min to achieve 45 °C, followed by cooling to 28 °C.

The synergistic effect of Dox and hyperthermia was next examined on HepG2 cells using live/dead staining assays. From Figure 6.23A, minimum cell death was observed when HepG2 cells were treated with one cycle of AMF (Figure 6.23A), indicating that in the absence of MNPs or DMCs, AMF-alone treatment was not toxic to the cells. In Figure 6.23B, minimum cell death was also observed when they were treated with 4-MC/NaIO₄ crosslinked DMCs in the absence of AMF, demonstrating that the leakage of Dox from DMCs was minimum and insufficient to induce HepG2 cell death. HepG2 cells treated with MNPs and one AMF cycle resulted in some HepG2 cell death due to the elevated temperature generated by MHT (Figure 6.23C). HepG2 cells treated with both 4-MC/NaIO₄ crosslinked DMCs and AMF resulted in some cell death and this amount of cell death increased with increasing number of AMF cycles (Figure 6.23D to F). After the 1st AMF cycle, the amount of Dox released from DMCs by MHT was insufficient to cause significant HepG2 cell death (15.3%, Figure 6.23D and 6.23G). Nevertheless, the 2nd AMF cycle resulted in substantial HepG2 cell death as an increase amount of Dox was released (93.1%, Figure 6.23E and 6.23G). Lastly, the 3rd AMF cycle resulted in 100% cell death (Figure 6.23F and 6.23G).

Next, the cytotoxicity of individual treatments (Dox only or MHT only) on HepG2 cells were studied. For MHT only treatment, MNPs-loaded crosslinked coacervates (without Dox) were added to HepG2 cells and subsequently subjected to 1 to 3 AMF cycles. From Figure 6.24A to C and 6.23G, the percentage of cell death increased from 12.6% to 65.9% with the number of MHT treatments. Despite the 3 MHT treatments, more than 30% of HepG2 cells had survived. For the study of Dox only treatment, crosslinked DMCs in cell culture media were first exposed to 1 to 3 cycles of AMF to induce Dox release. The DMCs within the cell culture were then removed and this cell culture media (which now contained the released Dox) was subsequently added to HepG2 cells. Like magnetic hyperthermia only treatment, Dox only treatment was not capable of killing all HepG2 cells (16.7% to 67.9%, Figure 6.24D to F and 6.23G). These data coincide with previous studies showing that thermo-chemotherapy is a more efficient cancer treatment when compared to chemotherapy only or hyperthermia only treatment.^{12, 56, 57}

Figure 6.23. Live/dead staining assay on treated HepG2 live cancer cells. (A) with one AMF cycle. (B) 4-MC/NaIO₄ crosslinked DMCs without AMF cycle. (C) MNPs with 1 AMF cycle (D-F) 4-MC/NaIO₄ crosslinked DMCs with 1 AMF cycle (D); 2 AMF cycles (E); and 3 AMF cycles (F). DMCs and MNPs concentrations were 500 $\mu\text{g/mL}$ and 0.04% v/v respectively. (G) Percentage of HepG2 cell death with different AMF cycles. ($n = 3$, mean $\pm$ S.D.) For MHT + Dox treatment after 3 cycles, 100% cell death was achieved.

Figure 6.24. Live/dead staining assay on MHT or Dox treated HepG2 live cancer cells. (A-C) MNPs-loaded, 4-MC/NaIO₄ crosslinked coacervates with 1 AMF cycle (A); 2 AMF cycles (B); and 3 AMF cycles (C). (D-F) Dox only treatment (Dox was first released from the 4-MC/NaIO₄ crosslinked DMCs in the cell culture medium before adding to cells) after 1 AMF cycle (D); 2 AMF cycles (E); and 3 AMF cycles (F). Concentration of DMC was 500 μg/mL.

6.4 Conclusion

In conclusion, DMCs have been developed for controlled release and thermo-chemotherapy treatments. These DMCs are cyto-compatible, magnetically responsive and can be uptake effectively into HepG2 liver cancer cells. This uptake of DMCs by HepG2 cells is not inhibited by chemical endocytosis inhibitors, signifying that DMC uptake is independent of conventional endocytosis pathways. Efficient uptake of DMCs by HepG2 cells allows the intracellular release of Dox within the cells instead of extracellular release, which allows the Dox to rapidly binds to DNA for its antitumor activity. Extracellular release of Dox are not preferred as the response time will be longer (time needed for diffusing across plasma membrane) and possibility of leakage to the surrounding healthy tissues. Dox release from DMCs at physiological temperature (37°C) is inadequate to induced HepG2 cell death whereas continuous release of Dox can be induced by heating through application of an external AMF. Combination of magnetic hyperthermia and Dox treatments (thermo-chemotherapy) act synergistically to increase the percentage of HepG2 cell death. As DMCs were cytocompatible, a large volume of DMCs can be given to the cancer patient and directed to the tumour site by magnetic targeting. Patient can then be

subjected to multiple cycles of AMF as needed. Since MHT and Dox can work synergistically, a lower dose of Dox is required when compared to intravenous injection of free Dox, resulting in better treatment efficacy and yet significantly lesser side effect. These results show that DMCs can be a propitious therapeutic modality in the treatment of liver cancer. Furthermore, the presence of MNPs within the DMCs open exciting opportunities in real-time magnetic resonance imaging and magnetic targeting for theragnostic approaches in treatment of liver cancer.

References

1. J. Balogh, D. Victor, 3rd, E. H. Asham, S. G. Burroughs, M. Boktour, A. Saharia, X. Li, R. M. Ghobrial and H. P. Monsour, Jr., *J Hepatocell Carcinoma*, 2016, **3**, 41-53.
2. A. S. Befeler and A. M. Di Bisceglie, *Gastroenterology*, 2002, **122**, 1609-1619.
3. A. Raza and G. K. Sood, *World J Gastroenterol*, 2014, **20**, 4115-4127.
4. M. JJG, *Journal of Carcinogenesis & Mutagenesis*, 2015, **06**.
5. C. Y. Liu, K. F. Chen and P. J. Chen, *Cold Spring Harb Perspect Med*, 2015, **5**, a021535.
6. K. Lohitesh, R. Chowdhury and S. Mukherjee, *Cancer Cell Int*, 2018, **18**, 44.
7. A. A. Argyriou, A. P. Kyritsis, T. Makatsoris and H. P. Kalofonos, *Cancer Manag Res*, 2014, **6**, 135-147.
8. T. Hashiguchi Y Fau - Fukuda, T. Fukuda T Fau - Ichimura, Y. Ichimura T Fau - Matsumoto, T. Matsumoto Y Fau - Yasui, T. Yasui T Fau - Sumi, O. Sumi T Fau - Ishiko and O. Ishiko.
9. P. M. Price, W. E. Mahmoud, A. A. Al-Ghamdi and L. M. Bronstein, *Front Chem*, 2018, **6**, 619.
10. J. Owen, Q. Pankhurst and E. Stride, *Int J Hyperthermia*, 2012, **28**, 362-373.
11. A. Hervault and N. T. Thanh, *Nanoscale*, 2014, **6**, 11553-11573.
12. M. Li, W. Bu, J. Ren, J. Li, L. Deng, M. Gao, X. Gao and P. Wang, *Theranostics*, 2018, **8**, 693-709.
13. G. K. Thirunavukkarasu, K. Cherukula, H. Lee, Y. Y. Jeong, I. K. Park and J. Y. Lee, *Biomaterials*, 2018, **180**, 240-252.

14. L. L. Lao and R. V. Ramanujan, *J Mater Sci Mater Med*, 2004, **15**, 1061-1064.
15. S. Purushotham and R. V. Ramanujan, *Acta Biomater*, 2010, **6**, 502-510.
16. S. Purushotham and R. V. Ramanujan, *Journal of Applied Physics*, 2010, **107**, 114701.
17. A. J. Cole, V. C. Yang and A. E. David, *Trends Biotechnol*, 2011, **29**, 323-332.
18. O. L. Gobbo, K. Sjaastad, M. W. Radomski, Y. Volkov and A. Prina-Mello, *Theranostics*, 2015, **5**, 1249-1263.
19. A. Singh and S. K. Sahoo, *Drug Discov Today*, 2014, **19**, 474-481.
20. Q. Zhang, W. Huang, C. Yang, F. Wang, C. Song, Y. Gao, Y. Qiu, M. Yan, B. Yang and C. Guo, *Biomater Sci*, 2019, **7**, 2729-2739.
21. R. Colombo, *Arch Ital Urol Androl*, 2008, **80**, 149-156.
22. R. Colombo, A. Salonia, Z. Leib, M. Pavone-Macaluso and D. Engelstein, *BJU Int*, 2011, **107**, 912-918.
23. B. Moskovitz, G. Meyer, A. Kravtsov, M. Gross, A. Kastin, K. Biton and O. Nativ, *Ann Oncol*, 2005, **16**, 585-589.
24. S. M. Carvalho, A. G. Leonel, A. A. P. Mansur, I. C. Carvalho, K. Krambrock and H. S. Mansur, *Biomater Sci*, 2019, **7**, 2102-2122.
25. H. C. Kim, E. Kim, S. W. Jeong, T. L. Ha, S. I. Park, S. G. Lee, S. J. Lee and S. W. Lee, *Nanoscale*, 2015, **7**, 16470-16480.
26. B. Liang, K. Yu, Y. Ling, M. Kolios, A. Exner, Z. Wang, B. Hu, G. Zuo, Y. Chen and Y. Zheng, *Biomater Sci*, 2019, **7**, 1815-1824.
27. B. T. Mai, P. B. Balakrishnan, M. J. Barthel, F. Piccardi, D. Niculaes, F. Marinaro, S. Fernandes, A. Curcio, H. Kakwere, G. Autret, R. Cingolani, F. Gazeau and T. Pellegrino, *ACS Appl Mater Interfaces*, 2019, **11**, 5727-5739.
28. F. Reyes-Ortega, A. V. Delgado, E. K. Schneider, B. L. Checa Fernandez and G. R. Iglesias, *Polymers (Basel)*, 2017, **10**.
29. J. Thevenot, H. Oliveira, O. Sandre and S. Lecommandoux, *Chem Soc Rev*, 2013, **42**, 7099-7116.
30. S. Y. Wang, M. C. Liu and K. A. Kang, *Adv Exp Med Biol*, 2013, **765**, 315-321.

31. L. G. Adeline Perro, Noémie Coudon, Sharvina Shanmugathan, Véronique Lapeyre, Bertrand Goudeau, Jean-Paul Douliez, Valérie Ravaine, *Journal of Colloid and Interface Science* 2019, **548**, 275-283.
32. W. C. Blocher and S. L. Perry, *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, 2017, **9**.
33. C. A. Gilroy, K. M. Luginbuhl and A. Chilkoti, *J Control Release*, 2016, **240**, 151-164.
34. J. M. Horn, R. A. Kapelner and A. C. Obermeyer, *Polymers (Basel)*, 2019, **11**.
35. D. S. Hwang, H. Zeng, A. Srivastava, D. V. Krogstad, M. Tirrell, J. N. Israelachvili and J. H. Waite, *Soft Matter*, 2010, **6**, 3232-3236.
36. N. R. Johnson, T. Ambe and Y. Wang, *Acta Biomater*, 2014, **10**, 40-46.
37. Z. W. Lim, Y. Ping and A. Miserez, *Bioconjug Chem*, 2018, **29**, 2176-2180.
38. K. A. Black, D. Priftis, S. L. Perry, J. Yip, W. Y. Byun and M. Tirrell, *ACS Macro Letters*, 2014, **3**, 1088-1091.
39. A. Nolles, A. H. Westphal, J. A. de Hoop, R. G. Fokkink, J. M. Kleijn, W. J. van Berkel and J. W. Borst, *Biomacromolecules*, 2015, **16**, 1542-1549.
40. N. K. Li, F. Garcia Quiroz, C. K. Hall, A. Chilkoti and Y. G. Yingling, *Biomacromolecules*, 2014, **15**, 3522-3530.
41. F. G. Quiroz and A. Chilkoti, *Nat Mater*, 2015, **14**, 1164-1171.
42. B. Vrhovski, S. Jensen and A. S. Weiss, *Eur J Biochem*, 1997, **250**, 92-98.
43. S. Kossatz, R. Ludwig, H. Dahringer, V. Ettelt, G. Rimkus, M. Marciello, G. Salas, V. Patel, F. J. Teran and I. Hilger, *Pharm Res*, 2014, **31**, 3274-3288.
44. E. A. Frankel, P. C. Bevilacqua and C. D. Keating, *Langmuir*, 2016, **32**, 2041-2049.
45. S. Lindhoud and M. M. Claessens, *Soft Matter*, 2016, **12**, 408-413.
46. J. Yang, M. A. Cohen Stuart and M. Kamperman, *Chem Soc Rev*, 2014, **43**, 8271-8298.
47. M. Kaksonen and A. Roux, *Nat Rev Mol Cell Biol*, 2018, **19**, 313-326.
48. H. Tang, Z. Gu, C. Li, Z. Li, W. Wu and X. Jiang, *Biomater Sci*, 2019, **7**, 2552-2558.
49. D. Dutta and J. G. Donaldson, *Cell Logist*, 2012, **2**, 203-208.

50. S. Vranic, N. Boggetto, V. Contremoulins, S. Mornet, N. Reinhardt, F. Marano, A. Baeza-Squiban and S. Boland, *Part Fibre Toxicol*, 2013, **10**, 2.
51. A. Akbari, A. Lavasanifar and J. Wu, *Acta Biomater*, 2017, **64**, 249-258.
52. M. Johannsen, U. Gneveckow, L. Eckelt, A. Feussner, N. Waldofner, R. Scholz, S. Deger, P. Wust, S. A. Loening and A. Jordan, *Int J Hyperthermia*, 2005, **21**, 637-647.
53. Q. A. Pankhurst, J. Connolly, S. K. Jones and J. Dobson, *Journal of Physics D: Applied Physics*, 2003, **36**, R167-R181.
54. S. Purushotham, P. E. Chang, H. Rumpel, I. H. Kee, R. T. Ng, P. K. Chow, C. K. Tan and R. V. Ramanujan, *Nanotechnology*, 2009, **20**, 305101.
55. R. E. Rosensweig, *Journal of Magnetism and Magnetic Materials*, 2002, **252**, 370-374.
56. C. Christie, S. Molina, J. Gonzales, K. Berg, R. K. Nair, K. Huynh, S. J. Madsen and H. Hirschberg, *Biomed Opt Express*, 2016, **7**, 1240-1250.
57. C. A. Quinto, P. Mohindra, S. Tong and G. Bao, *Nanoscale*, 2015, **7**, 12728-12736.

Chapter 7

Discussion and future work

This Chapter discuss the significant findings of Chapter 4 to 6, regarding DgHBP-2 peptide coacervates and its integration into GRIDS and thermo-chemotherapy. Preliminary studies on therapeutic peptides and RNA-RFP encapsulation, uptake studies using GUVs and in vivo biodistribution studies are also described in this Chapter. In addition, recommendations for future work on peptide coacervates are proposed in this chapter.

7.1 General discussion

As a fully organic biotool with outstanding mechanical properties, the squid beak of *D.gigas* has attracted the attention of many material scientists.¹⁻³ Although previous work had revealed the relationship between its mechanical gradient and composition, along with identification and physico-chemical transitions of the beak proteins, the application of these beak proteins into biomimetic design has never been done.^{1, 4-6} Among the two families of beak proteins (DgCBPs and DgHBPs), only DgHBPs have been shown to undergo coacervation.^{1, 4} Since encapsulation is an important application of coacervation, this project aims to utilise biomimetic coacervates derived from beak proteins of *D.gigas* to fabricate different stimuli-responsive drug delivery system. This project has provided a comprehensive understanding of DgHBP-based biomimetic peptides coacervation, different crosslinking methods and its application into glucose/temperature responsive delivery systems.

7.1.1 Characterisation of DgHBP-2 peptide coacervates

In Chapter 4, the coacervation properties of DgHBPs and biomimetic peptides has been extensively studied using turbidimetry and light microscopy. From the five tested proteins and peptides, only DgHBP-2 peptide was used for further studies because of its ability to undergo coacervation at physiological pH and ionic strength (Table 4.1). DgHBP-2 peptide coacervates are pH responsive (pH 7.5 to 9.5), making it an attractive replacement for synthetic pH sensitive polymers. With the replacement of GHGX₁X₂, to GAGFA/GFA motifs, the level of coacervation decreases at lower ionic strength and pH range becomes narrower. Hence, by changing the sequence and/or its length, the pH range, ionic strength and thermo-responsiveness in theory can be tuned according to different requirements.

While the tandem repeats on DgHBPs are similar to those of tropoelastin^{1, 4}, heating triggered DgHBP-2 peptide coacervates dissociation instead of coacervation. This heat-triggered dissociation allowed the DgHBP-2 peptide coacervates to release its drug contents at a rate tuneable through the control of external heat application e.g. magnetic hyperthermia. This have advantage over tropoelastin or ELP where drug release can be combined with hyperthermia for a synergistic therapeutic effect. Therefore, DgHBP based

peptides are a novel class of pH- and thermo-responsive materials for biomedical applications.

As DgHBP-2 coacervates merged upon contact with each other, the size of coacervates increased overtime. This has implications on the drug release kinetics where smaller particles are known to release drug faster than larger particles.⁷⁻⁹ Different crosslinking mechanisms for the coacervates have been explored for decreasing coalescence between coacervates and for washing off excess unencapsulated drugs. Since the rostral regions within the squid beak have been proven to contain crosslinked catechol-rich compounds^{2,6}, catechol-containing unnatural amino acid, DOPA is used to replace all the 5 tyrosine. On the other hand, 4-MC is added exogenously to mimic catechol molecules secreted by beccublast cells. Coacervates are liquid-like microdroplets that undergo Ostwald ripening or coalescence of droplets, resulting in a large size distribution. The exact reason behind the change in size distribution after crosslinking (two size population) is currently unknown and will be tested in the future. The crosslinking process may have reduced the extent of Ostwald ripening and fusion of coacervates that contributes to this bimodal size population. However, this theory remains to be tested. DOPA-Fe crosslinked coacervates are stable for a wider range of pH (between pH 5 to 9.5) due to its pH sensitive catechol-Fe coordination bonds. This reversible crosslinked coacervates can be useful for pH responsive drug delivery mechanism. By changing the crosslinker from FeCl₃ to NaIO₄, the final crosslinked coacervates can be suitable for oral delivery of therapeutics because of its stability in extreme pH conditions.

In vitro cytotoxicity assays (MTT assay and Live/dead staining) were done on DgHBP-2 peptide, un-crosslinked coacervates, crosslinked coacervates and found to be cytocompatible. While biodegradable polymers are known to be non-toxic, the safety of its degradation products is in question.¹⁰ In contrast, the degradation of peptides results in the release of amino acid residues which are ubiquitous and nontoxic.

7.1.2 Glucose responsive insulin delivery system

In Chapter 5, a GRIDS fabricated by co-encapsulation of GOx and insulin into DgHBP-2 peptide coacervates was presented. It is made of entirely natural materials and

is free of electronics that are needed for existing closed-loop artificial pancreas. The coacervates are made from biomimetic peptides that are non-toxic, cheap to produce and easily-modifiable. In this GRIDS, GOx is chosen as the glucose sensor instead of boronic acids or GBPs. While PBA-based insulin delivery vehicles are also glucose-responsive¹¹⁻¹⁴, it is challenging to optimise them such that they remained glucose-responsive under physiological pH and temperature.¹⁵⁻¹⁷ This is because the binding affinity of PBA to glucose only increases when the pH is above the pKa of PBAs (~8 to 9).¹⁸ Also, the formation of boronate esters are not limited to glucose, allowing the unspecific triggered release of insulin. Multivalent-boronic acids that have been developed for specific glucose binding are not suitable to be used as a crosslinker, due to their specific configuration of boronic acids.¹⁹ On another hand, GBPs are highly immunogenic due to its bacteria origin and may not be suitable for *in vivo* biomedical applications.^{20, 21} Hence, GOx is used in this GRIDS instead of PBA or GBP.

The oxidation of glucose to gluconic acid consumes oxygen and results in the production of toxic intermediate H₂O₂, hence the limiting factors for GOx are the lack of oxygen and build-up of H₂O₂.²¹ In order to overcome these limiting factors, catalase have been integrated into other GRIDS to break down H₂O₂ into oxygen.^{22, 23} Yet, catalase is not needed in the DgHBP-2 peptide coacervation-based GRIDS, as shown from the results of *in vitro* insulin release assay. This is because coacervates allow the rapid passive diffusion of small molecules across its boundary while selectively compartmentalising other larger molecules.^{24, 25} This simplifies the GRIDS fabrication process, allowing more insulin and GOx to be packaged into the coacervates. As the glucose concentration increases, the rate of glucose diffusion into the coacervates increases, resulting in a higher production rate of gluconic acid. When the rate of gluconic acid production is faster than the rate of gluconic acid diffusing out of the coacervates, gluconic acid accumulates within the coacervates resulting in a decreased localised pH that dissociates the coacervates and ultimately release of insulin. Electrostatic repulsion between the negatively charged gluconic acid (pKa: 3.7) and insulin (pI 5.3) within the coacervates may have also contributed to the latter release. However, this remains to be tested. Many research has been done to improve insulin release algorithm of closed-loop delivery systems, as their

insulin release action is often delay due to differences between blood and interstitial glucose concentrations.^{26,27} Therefore, rapid diffusion of glucose in and out of coacervates allow the GRIDS to respond promptly to changes in glyucose concentration.

In this system, >99% of the insulin was encapsulated successfully, irrespective of FITC attachment. The reason behind the extremely high insulin EE% by the coacervates is still unknown. Nonspecific interactions among insulin and the peptide are most likely to be involved, particularly π - π and hydrophobic interactions because of the large percentage of Tyr and hydrophobic residues within the peptide, but this remains to be confirmed. Since close to 100% of the insulin had been encapsulated, removing the unencapsulated insulin was unnecessary and the stabilisation of coacervates by crosslinking can be omitted. This high loading capacity coupled with low toxicity of DgHBP-2 peptide coacervates reduce the need for frequent dosing in diabetic patients, which can be several times per day for current insulin replacement therapy.

From the cell uptake studies shown in chapter 6, it is likely that the coacervates based GRIDS will also be uptake by the cells. While cellular uptake was preferred in other drug delivery systems, it is not preferred in GRIDS as insulin binds to extracellular receptors. The addition of PEG to liposomes and nanoparticles has been shown to prevent uptake by the RES system and thus improving the circulation time.²⁸ Hence, PEGylation of DgHBP-2 peptide can be done similarly to prevent cellular uptake, provided that it does not interfere with the coacervation process. Another possible way to improve the circulation time is by coupling the coacervates to the surface of red blood cells (RBCs), a process commonly known as RBC-hitchhiking.²⁹⁻³¹ RBCs have been studied intensively in recent years to be used as carrier, due to its long systemic circulation (~120 days), flexibility, inherent biocompatibility and low immunogenicity. While RBC-hitchhiking has never been tested on coacervates, it has been shown to significantly improve the blood circulation time of nanoparticles. The addition of RBCs (long circulation time and low immunogenicity) into the coacervate based GRIDS (large insulin capacity and glucose sensing) will further improve the latter's therapeutic efficacy for the treatment of diabetes.

CD spectroscopy and ELISA were used in this study to infer the bioactivity of insulin. However, these assays do not unequivocally prove that the activity of insulin is maintained. A cell-based assay that can directly measures insulin-triggered response will be needed to demonstrate insulin activity. An example of a cell-based assay is iLite® Insulin Assay Ready Cells (Svar Life Science, Sweden), which are genetically engineered cells that can express Firefly luciferase under an insulin responsive promoter. Upon successful binding to the insulin receptor, downstream signalling results in the expression of luciferase reporter gene. The amount of luciferase signal, which is proportional to the amount of insulin, can then be quantified in a luminometer after the addition of luciferase substrate.

7.1.3 Magnetically Responsive Peptide Coacervates with Dual Triggered Hyperthermia and Chemotherapy for Liver Cancer Treatment

Liver cancer is an aggressive malignancy that is difficult to treat. While hepatic resection is an effective therapy for liver cancer, many patients still suffer from cancer recurrence and metastases (up to 78%).^{32, 33} Residual cancerous cells after surgery contribute to the high recurrence rate. Also, liver cancer is highly chemotherapy refractory, making chemotherapy only slightly effective in shrinking the tumour size. Hyperthermia is a type of cancer therapy where heat is used to inhibit the growth or kill cancer cells. As a monotherapy, hyperthermia showed only limited efficacy in cancer treatment.³⁴⁻³⁶ When combined with chemotherapy, hyperthymia have been shown to have a synergistic effect in improving the cytotoxic effects of chemotherapy.³⁷⁻⁴²

In chapter 6, Dox and MNPs are co-encapsulated within DgHBP peptide coacervates (DMCs) for thermo-chemotherapy of liver cancer. Dox served as a model chemotherapeutic drug while MNPs was used for targeting and MHT. Results from *in vitro* cytotoxic assays are in line with previous studies, showing that combined therapy is more effective in inducing cell death when compared to chemotherapy alone or hyperthermia alone therapy. While these DMCs were not tested *in vivo*, 100% HepG2 cell death was achieved in cell culture studies suggesting that combined therapy may help to prevent recurrence of liver cancer. These crosslinked DMCs are tested to be stable for at least 15

days and cytocompatible in the absence of AMF, allowing them to be used as a Dox reservoir within body for longer period of time, triggering Dox release only when needed. Hence, patients can be administrated with larger dose of DMCs at a lesser frequency, improving patient compliance and reducing infection risk at the same time.

Despite large size of DgHBP-2 peptide coacervates, they were uptaken efficiently into HepG2 liver cancer cells. The cellular uptake mechanism of coacervates in mammalian cells has never been studied. By using chemical inhibitors, the uptake of DgHBP-2 coacervates is revealed to be likely in an endocytosis- and ATP-independent process. The exact mechanism of uptake is currently unknown and maybe related to the liquid-like nature of coacervates. Even after crosslinking, DgHBP-2 peptide coacervates still exhibit fluidic-like characteristics as shown in TEM. One possible uptake mechanism is by facilitated diffusion, which uses carrier proteins or protein channels to transport target molecules from outside cell into other side of membrane. Even though the size of coacervates are much bigger than molecules using facilitated diffusion, coacervates are liquid-based and may deform to pass through the protein channels/carrier proteins. Another possible uptake mechanism is related to the ultra-low interfacial tension and good wetting properties of coacervates.⁴³⁻⁴⁵ These properties may allow coacervates to spread over the cell membrane, which eventually allow it to pass through the cell membrane.

7.2 Reconnaissance work for future studies

Preliminary studies on other biomedical applications and cellular uptake mechanism of DgHBP-2 peptide coacervates will be presented in this chapter. These results will be used as a basis for future work shown in Chapter 7.3.

7.2.1 Encapsulation of therapeutic peptides

Therapeutic peptides have become increasingly important in the treatment of various disease such as infections and cancers.^{46, 47} However, they are often unable to penetrate cell membrane efficiently and are unstable within the blood circulation.^{48, 49} Thus, there is a pressing need to develop new methods of delivering these therapeutic peptides. Current strategies to improve membrane penetration include fusion with cell-penetrating peptides

or protein transduction domain. Nonetheless, these processes are labour intensive and may eventually result in the loss of bioactivity. Since DgHBP-2 peptide coacervates have been successfully used to delivery insulin and Dox, the encapsulation of therapeutic peptides was next tested.

The p53 protein is a tumour suppressor protein which helps to prevent the formation of tumours by arresting cell cycle and inducing apoptosis.^{50, 51} Because of this important role, p53 protein is often inactivated in cancer cells, either by mutations within the p53 protein or by mutations in the regulatory proteins of p53 pathway. Despite the expression of wildtype p53 remains unaffected in ~ 50% of the cancers, its activation pathways were often repressed in these cancers.⁵² Mouse double minute 2 homolog (MDM2) protein is a ubiquitin-protein ligase that negatively regulates p53 protein by promoting its degradation.⁵³ By blocking the interaction between p53 and MDM2 (a negative regulator of p53), p53 signalling within the tumours can then be restore, leading to inhibition of cell growth and ultimately cell death. Hence, peptides that can selectively inhibit interactions between p53 and MDM2 are of great interest.^{52, 54} Encapsulation of these MDM2-targeting peptides (derived from N-terminal region of p53 sequence) can be used to improve its stability against protease degradation and cell membrane permeability. Two different MDM2-targeting peptides (PM-2 and VIP-71) had been encapsulated into DgHBP-2 peptide coacervates (Figure 7.1). The encapsulation efficiency of PM-2 and VIP-71 were 100.0 % and ~71.5 % respectively. This suggests that coacervates can be used as a new encapsulation materials for delivery of therapeutic peptides.

Figure 7.1. RP-HPLC chromatogram (220nm) of (A) PM-2 peptide (0.14 mg/mL); (B) supernatant after removing PM-2 loaded coacervates; (C) VIP-71 peptide (0.37 mg/mL) and (D) supernatant after removing VIP-71 loaded coacervates.

7.2.2 Encapsulation of CRISPR-Cas9 system

Clustered Regularly Interspace Short Palindromic Repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) is a genome editing system derived from the adaptive immunity of some archaea and bacteria.⁵⁵ CRISPR-Cas 9 system consists of two components, guide RNA and Cas9 protein. CRISPR-Cas9 has been widely used in genetic manipulation such as knock-in or knock-out of genes, transcriptional repression and activation.⁵⁶ While efficient genomic manipulation has been accomplished in some cancer cell lines,^{57, 58} manipulation of stem cells has been challenging due to large number of cell death after genomic editing.⁵⁹ Currently, the most efficient way of delivering CRISPR-Cas9 system is through electroporation, which can cause further cell death. Since coacervates are non-

toxic and can be uptake into the cells efficiently, delivery of CRISPR-Cas 9 system was next tested. RNA conjugated to Red fluorescence protein (RFP) was used in place of CRISPR-Cas 9 system was encapsulated within DgHBP-2 peptide coacervates and delivered into human induced pluripotent stem cells (iPSCs). From Figure 7.2B and D, RNA-RFP loaded coacervates were uptake into iPSCs without causing any significant cell death, suggesting that coacervates may be superior than electroporation in the delivery of CRISPR-Cas 9 system.

Figure 7.2. Micrographs of human iPSCs after treated with RNA-RFP loaded coacervates. (A) 3 hours control (without coacervates); (B) Coacervates treated cells (3 hours); (C) 24 hours control (without coacervates) and (D) Coacervates treated cells (24 hours).

7.2.3 Uptake mechanism of DgHBP-2 peptide coacervates

In Figure 6.13 to 6.16, crosslinked coacervates could be uptake into HepG2 cells through an endocytosis- and ATP-independent pathway. To further understand the uptake mechanism, the uptake of coacervates into giant unilamellar vesicles (GUVs) was next

studied. GUVs are made of phospholipids, which have two lipophilic tails chains and one hydrophilic head group. The membrane of GUVs is consisted of a single bilayer, where the hydrophilic head groups of outer layer are facing outwards and hydrophilic head groups of inner layer are facing interior of the GUVs. The GUVs in these experiments were made from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), a type of phosphatidylcholine that are naturally present in mammalian cell membranes and has been previously used for cellular uptake studies.^{60, 61} In Figure 7.3, GFP-loaded crosslinked coacervates were associated with the GUVs, with a layer of membrane that appeared to be wrapped around the coacervates. However, no coacervates were found inside the GUV. Since the coacervates are able to uptake by HepG2 cells and can only associate with lipid bilayer of GUV, this suggests that interaction with POPC alone is not sufficient for uptake of coacervates.

Figure 7.3. Interaction between GUV (doped with 0.1 mol% Rhodamine-PE) and GFP-loaded 4-MC/NaIO₄ crosslinked coacervates in 100 mM glucose solution. Left: 3D projection of deconvoluted z-stack. Right: 2D cross-sectional views of different channels.

Next, the uptake of DgHBP-2 peptide itself was tested. This is to determine if DgHBP-2 peptide molecules or its coacervation phenomenon is important for cellular uptake. A FITC was attached to the DgHBP-2 peptide at the N-terminal (FITC-DgHBP-2 peptide), resulting in a loss of coacervation properties. Interestingly, FITC-DgHBP-2

peptide did not associate with lipid bilayer nor entered the GUV (Figure 7.4), suggesting that the coacervation properties is important for the cellular uptake. FITC-DgHBP-2 peptide was next tested on HepG2 cells to determine its cellular uptake properties. Similarly, FITC-DgHBP-2 peptide was not uptaken into HepG2 cells (Figure 7.5).

Figure 7.4. Micrographs of FITC-DgHBP-2 peptide with GUVs. (A) Merged channel; (B) Red fluorescence and (C) Green fluorescence.

Figure 7.5. Confocal micrographs (orthographic view) of HepG2 cells treated with (A) 1 μ M and (B) 10 μ M of FITC-DgHBP-2 peptide for 24 hours.

7.2.4 *In vivo* biodistribution studies of coacervates

Biodistribution studies are the methods of following where the targeted molecules travel in a human or animal subject. It is crucial in understanding the target organs involved in addition predicting efficacy and safety. In order to track the coacervates inside wildtype mouse, Cyanine (Cy) 5.5 NHS ester was loaded into coacervates (Figure 7.6). From Figure 7.7, most of the coacervates were cleared rapidly by the liver, kidney and spleen within 1 hour. By 24 hours, the fluorescence became comparable to organs of control mouse (un-injected). To improve the duration of coacervation circulation, surface modification of coacervates can be done. One possible way of surface modification is through coating of serum proteins such as albumin onto the surface of coacervates. Indeed, BSA coating have been shown to improve plasma circulation time and cellular uptake.⁶²⁻⁶⁴ To determine if BSA-FITC could be deposited on to coacervates surface, it was added to cy5.5-loaded coacervates directly and then view under confocal microscopy. BSA-FITC were absorbed to the coacervates surface upon contact (Figure 7B and D), mostly likely through non-specific interactions. More studies are required to apprehend the outcome of BSA coated coacervates in the blood circulation and cellular uptake.

Figure 7.6. Micrographs of cy5.5-loaded coacervates. Fluorescence micrograph (left); Optical micrograph (middle) and Merged channel micrograph (right).

Figure 7.7. Total radiant of BL6 mouse organs injected with 100 μ L of cy5.5-loaded coacervates at 1- and 24-hour post-injection. Un-injected wildtype BL6 mice act as control.

Figure 7.8. confocal micrographs of BSA-FITC coated, cy5.5-loaded coacervates. (A and B) 3D projection of z-stack, (C) Z stack image and (D) orthographic view. Green fluorescence and red fluorescence represent BSA-FITC and cy5.5-loaded coacervates respectively.

7.3 Outlook and Future work

Biomimetic peptide coacervates have so far shown to exhibit excellent cytocompatibility and able to encapsulate a wide range of molecules. The next step of action would be to study these coacervates *in vivo* for exploring their possible applications in biomedical and bioengineering fields. Nowadays, peptides can be synthesised easily from amino acids with the use of automated peptide synthesizers. Hence, high throughput screening of different peptide sequences and large-scale production of peptides can be easily achieved. The potential applications of these peptides can expand beyond drug delivery vehicles shown in this project, to non-biomedical applications such as underwater adhesives and enzymatic reaction vessels.

7.3.1 Relationship between peptide sequences, length and coacervation properties

In this project, only two of the biomimetic peptides (DgHBP-2 and pepHBP-1) were tested. Hence, it will be crucial to understand the relationship between the number and type of tandem repeats with the coacervation pH range and temperature stability. Once this information is established, an algorithm can be put in place to generate polypeptides of known coacervation properties and stabilities. This algorithm can be useful for generating polypeptides for pH sensitive applications such as oral delivery of therapeutics. Also, the length and sequences of peptide may have a role in controlling the therapeutics release kinetics.

While DgHBP-2 peptide coacervates are able to encapsulate a range of molecules, the exact mechanism of interaction between the peptide and its target molecules have not been explored yet. Understanding the underlying mechanism will be useful for the optimisation of therapeutics encapsulation.

7.3.2 *In vivo* biodistribution studies of coacervates

DgHBP-2 peptide coacervates are rapidly cleared by the reticuloendothelial system (RES), reducing their bioavailability and half-life in the circulation. Fast clearance will also reduce the amount of coacervates reaching the target site. Since BSA- or PEG-coated nanoparticles have shown to improve circulation time,^{62, 65-67} biodistribution of coated coacervates shall be tested and compared to non-coated coacervates.

Magnetic coacervates align themselves into strings of coacervates under directional magnetic field, signifying that magnetic targeting is possible *in vivo*. By using directional magnetic field from permanent magnets, the magnetic coacervates can be attracted towards the target organ and decrease its clearance rate. The effectiveness of magnetic targeting can be evaluated by viewing at histological sections of the target organ.

7.3.3 Diabetic animal models studies

Animal studies have historically played an important role in biomedical research such as understanding disease pathology, assessment of novel therapeutics and treatment

routines.⁶⁸ Type 1 and 2 diabetes are the two main types of diabetes and different animal models have been developed for these two type of diabetes. Type 1 diabetic animal models are usually developed from either spontaneously autoimmune diabetes or chemically induced diabetes through ablation of insulin-producing cells (beta cells).^{68, 69} Type 2 diabetic animal models are mainly developed from induced obesity or impairment of beta cell by genetically modification.^{68, 69} While there are more people suffering from type 2 diabetes, the coacervates-based GRIDS is a type of insulin replacement therapy and therefore should be tested on type I mouse models. GRIDS can be injected into type I mouse model, followed by monitoring of blood glucose for a period of time. If the GRIDS is effective, the blood glucose will be decreased in the treated mouse when compared to the controls (untreated).

The use of chemicals such as streptozotocin and alloxan is a simple and cheap way to generate type 1 diabetic mouse model. These chemicals are toxic analogs of glucose and are rapidly uptaken by beta cells, eventually leading to cell death.⁶⁹ The main disadvantage of using these chemicals are the potential toxicity towards other organs and hence great care must be taken in controlling the dosage.⁶⁹ The most common spontaneous autoimmune model used is the non-obese diabetic (NOD) mouse.^{69, 70} NOD mouse suffers from insulinitis by 4 weeks old, leading to beta cell death and loss of insulin production. However, the onset of diabetes is slow (10 to 4 weeks) and can be as long as 30 weeks. Also, female mice are more likely to develop diabetes (up to 90%) when compared to male mice (up to 30%).⁷¹ Diabetes development in NOD mice have been shown to decrease with microbial exposure, thus they need to be raised in specific pathogen free (SPF) conditions to sustain their diabetic occurrence.⁶⁹ Because of the unpredictability in disease incident and the need for SPF conditions, NOD mice are more expensive to maintain when compared to chemically induced diabetic mice.

7.3.4 Liver cancer animal model

Creating animal model for liver cancer is important for both fundamental and applied research.⁷² Laboratory mouse is one of the recommended animal model due to genetic and molecular similarities to human, fast breeding cycle and ease of genetic

manipulation. Since liver is the primary target site for many chemicals, hepatocarcinogenic compounds such as diethylnitrosamine (DEN) can be used to encourage liver cancer in mouse.⁷³ DEN is metabolised by cytochrome p450 enzymes within hepatocytes and becomes carcinogenic. Young mice (less than 2 weeks) are highly susceptible while older mice require tumour promotion in the form of additional chemicals e.g. phenobarbital or high fat diet feeding.^{72,74} DEN is a DNA alkylating agent that cause breakages on DNA strands resulting in sequence mutation. Also, bioactivation of DEN by hepatocytes produces reactive oxygen species, leading to further DNA damage and cell death. Besides chemically induced liver cancer, transgenic mouse lines containing integrated hepatitis B or C virus genome, individual viral genes have also been established.⁷⁵

Although closely related to humans, mouse models can still result in a different therapeutic response. Chronic exposure of carcinogen can also be expensive and time consuming. To better evaluate therapeutic potentials of drugs and mimic human tumour microenvironment, xenograft models are established by implanting human cancer cells into immune deficiency mice.⁷⁶ Depending on their implantation site, xenograft models are classified into ectopic or orthotopic tumour models.⁷⁶ Ectopic tumour models are when the implantation site is differs from the source of the human tumour cells. Implantation site is usually located at the subcutaneous tissue of hind leg or dorsal region of mice. This method is frequently used for assessment of therapeutic methods because tumour growth and tumorigenicity can be easily monitored.⁷⁷ However, this method cannot be used for studying metastasis as the microenvironment is already different from the implanted tumour cells.⁷⁶ Orthotopic models are when the implantation site is same as the source of the human tumour cells. Such models are difficult to generate and assess as most tumours are imperceptible using naked eye. Therefore, it is not suitable for routine assessment of anticancer activity.

7.3.5 Cellular uptake mechanism of coacervates

From Chapter 6, the uptake mechanism of coacervates appeared to be endocytosis- and ATP-independent. Preliminary studies using GUVs had shown that while the coacervates can associate with POPC-based lipid bilayer, it could not enter the GUV. 1%

to 5% of charged lipids (that has opposite charge to coacervates) could be added to GUVs. The attractive force between the charged lipids and coacervates may help the latter to penetrate GUV. Since the majority of structure lipids on mammalian membranes are glycerophospholipids and sphingolipid,⁷⁸ GUV comprising a mixture of these lipids can be tested next. The effect of cholesterol can also be tested using cholesterol-containing GUVs.⁷⁹

In Figures 6.13 and 6.14, GFP-loaded crosslinked coacervates exhibited puncta staining pattern surrounding the nucleus. Since endoplasmic reticulum and golgi apparatus are two of the organelles that are located at the periphery of the nucleus, the coacervates maybe associated with or reside within these organelles. To confirm this hypothesis, co-localisation studies between the coacervates and organelles can be done.

7.3.6 Peptide delivery and Transfection studies

Other than insulin, DgHBP-2 peptide coacervates are capable of encapsulating other therapeutic peptides such as MDM2-targeting peptides. The encapsulation of MDM2-targeting peptides can improve its cellular uptake, half-life and resistance to proteases cleavage. Since encapsulation of MDM2-targeting peptide is successful, the next step will be treating MDM2-targeting peptide loaded coacervates on cancer cells that overexpresses MDM2. Overexpression of MDM2 can be achieved by transfection of MDM2 cDNA containing plasmid or transgenic overexpression.^{80, 81} Alternatively, cancer cell lines that have acquired overexpression of MDM2 can also be used.⁸²⁻⁸⁴ Overexpression of MDM2 in cells decreases p53 protein levels, leading to increase proliferation and survival.⁸⁵ MDM2-targeting peptide loaded coacervates once uptake by cancer cells will prevent the degradation of p53, leading to decrease proliferation and increased cell death.

Similarly, coacervates can be used to deliver CRISPR-Cas9 system into cells for genetic manipulation. Current methods of transfection include lipofection, electroporation, viral transfection and microinjection.⁸⁶ Lipofection has low toxicity but are not as efficient as other methods. Electroporation is more efficient than lipofection but often result in high number of cell death.⁸⁷ Viral transfection is compatible to various cell types but is time

consuming and immunogenic. Microinjection has the highest efficiency but is extremely labour-intensive and can only be low-throughput. Hence, the use of coacervates for delivering CRISPR-Cas9 system can be a new addition to the current methods of transfection.⁸⁶

Reference

1. H. Cai, B. Gabryelczyk, M. S. S. Manimekalai, G. Gruber, S. Salentinig and A. Miserez, *Soft Matter*, 2017, **13**, 7740-7752.
2. A. Miserez, T. Schneberk, C. Sun, F. W. Zok and J. H. Waite, *Science*, 2008, **319**, 1816-1819.
3. A. Miserez and J. H. Waite, 2010, DOI: 10.1036/1097-8542.yb100059.
4. Y. Tan, S. Hoon, P. A. Guerette, W. Wei, A. Ghadban, C. Hao, A. Miserez and J. H. Waite, *Nat Chem Biol*, 2015, **11**, 488-495.
5. A. Miserez, Y. Li, J. H. Waite and F. Zok, *Acta Biomater*, 2007, **3**, 139-149.
6. A. Miserez, D. Rubin and J. H. Waite, *J Biol Chem*, 2010, **285**, 38115-38124.
7. N. Y. Abu-Thabit and A. S. H. Makhoulouf, in *Stimuli Responsive Polymeric Nanocarriers for Drug Delivery Applications, Volume 1*, eds. A. S. H. Makhoulouf and N. Y. Abu-Thabit, Woodhead Publishing, 2018, DOI: <https://doi.org/10.1016/B978-0-08-101997-9.00001-1>, pp. 3-41.
8. D. Loca, J. Locs, A. Dubnika, V. Zalite and L. Berzina-Cimdina, in *Hydroxyapatite (Hap) for Biomedical Applications*, ed. M. Mucalo, Woodhead Publishing, 2015, DOI: <https://doi.org/10.1016/B978-1-78242-033-0.00009-2>, pp. 189-209.
9. Z. P. Aguilar, in *Nanomaterials for Medical Applications*, ed. Z. P. Aguilar, Elsevier, 2013, DOI: <https://doi.org/10.1016/B978-0-12-385089-8.00005-4>, pp. 181-234.
10. W. B. Liechty, D. R. Kryscio, B. V. Slaughter and N. A. Peppas, *Annu Rev Chem Biomol Eng*, 2010, **1**, 149-173.
11. D. Shiino, Y. Murata, A. Kubo, Y. J. Kim, K. Kataoka, Y. Koyama, A. Kikuchi, M. Yokoyama, Y. Sakurai and T. Okano, *Journal of Controlled Release*, 1995, **37**, 269-276.

12. D. H. Chou, M. J. Webber, B. C. Tang, A. B. Lin, L. S. Thapa, D. Deng, J. V. Truong, A. B. Cortinas, R. Langer and D. G. Anderson, *Proc Natl Acad Sci U S A*, 2015, **112**, 2401-2406.
13. G. Wu, C. Li, X. Liu, J. Lv, Y. Ding, Y. Liu, Y. Liu, F. Huang, L. Shi, Y. An and R. Ma, *Colloids Surf B Biointerfaces*, 2019, **180**, 376-383.
14. J. Wang, J. Yu, Y. Zhang, X. Zhang, A. R. Kahkoska, G. Chen, Z. Wang, W. Sun, L. Cai, Z. Chen, C. Qian, Q. Shen, A. Khademhosseini, J. B. Buse and Z. Gu, *Sci Adv*, 2019, **5**, eaaw4357.
15. A. Matsumoto, K. Yamamoto, R. Yoshida, K. Kataoka, T. Aoyagi and Y. Miyahara, *Chem Commun (Camb)*, 2010, **46**, 2203-2205.
16. Y. Zhang, Y. Guan and S. Zhou, *Biomacromolecules*, 2006, **7**, 3196-3201.
17. L. Zhao, Q. Huang, Y. Liu, Q. Wang, L. Wang, S. Xiao, F. Bi and J. Ding, *Materials (Basel)*, 2017, **10**, 170.
18. M. Elsherif, M. U. Hassan, A. K. Yetisen and H. Butt, *ACS Nano*, 2018, **12**, 2283-2291.
19. W. Zhai, L. Male and J. S. Fossey, *Chem Commun (Camb)*, 2017, **53**, 2218-2221.
20. G. Tiegs, J. Hentschel and A. Wendel, *J Clin Invest*, 1992, **90**, 196-203.
21. M. Rigla Cros, *Endocrinol Nutr*, 2016, **63**, 143-144.
22. M. Y. Kim and J. Kim, *ACS Biomaterials Science & Engineering*, 2017, **3**, 572-578.
23. Z. Gu, A. A. Aimetti, Q. Wang, T. T. Dang, Y. Zhang, O. Veis, H. Cheng, R. S. Langer and D. G. Anderson, *ACS Nano*, 2013, **7**, 4194-4201.
24. A. A. Hyman, C. A. Weber and F. Julicher, *Annu Rev Cell Dev Biol*, 2014, **30**, 39-58.
25. B. Drobot, J. M. Iglesias-Artola, K. Le Vay, V. Mayr, M. Kar, M. Kreysing, H. Mutschler and T. D. Tang, *Nat Commun*, 2018, **9**, 3643.
26. R. Hovorka, *Diabet Med*, 2006, **23**, 1-12.
27. A. N. Zaykov, J. P. Mayer and R. D. DiMarchi, *Nat Rev Drug Discov*, 2016, **15**, 425-439.
28. M. J. Roberts, M. D. Bentley and J. M. Harris, *Adv Drug Deliv Rev*, 2002, **54**, 459-476.

29. E. Chambers and S. Mitragotri, *Exp Biol Med (Maywood)*, 2007, **232**, 958-966.
30. X. Han, C. Wang and Z. A.-O. Liu.
31. C. H. Villa, A. C. Anselmo, S. Mitragotri and V. Muzykantov, *Adv Drug Deliv Rev*, 2016, **106**, 88-103.
32. H. J. Wanebo, Q. D. Chu, K. A. Avradopoulos and M. P. Vezeridis, *Surgery*, 1996, **119**, 361-371.
33. N. Portolani, A. Coniglio, S. Ghidoni, M. Giovanelli, A. Benetti, G. A. Tiberio and S. M. Giulini, *Ann Surg*, 2006, **243**, 229-235.
34. T. S. Herman, M. Teicher Ba Fau - Jochelson, J. Jochelson M Fau - Clark, G. Clark J Fau - Svensson, C. N. Svensson G Fau - Coleman and C. N. Coleman, 1988.
35. H. T. Newsam Je Fau - Law and H. T. Law, 1982.
36. J. L. Meyer, *Cancer Res*, 1984, **44**, 4745s-4751s.
37. B. Moskovitz, G. Meyer, A. Kravtsov, M. Gross, A. Kastin, K. Biton and O. Nativ, *Ann Oncol*, 2005, **16**, 585-589.
38. M. Li, W. Bu, J. Ren, J. Li, L. Deng, M. Gao, X. Gao and P. Wang, *Theranostics*, 2018, **8**, 693-709.
39. R. E. Falk, L. Newhook, F. L. Moffat, W. B. Lopatin, J. A. Heine, L. Makowka, V. Howard, D. Laing, D. Venturi and J. A. Falk, *Recent Results Cancer Res*, 1986, **100**, 315-320.
40. I. Tohnai, Y. Hayashi, K. Mitsudo, T. Kobayashi, M. Matsui and M. Ueda, *Nagoya J Med Sci*, 1996, **59**, 49-54.
41. I. Tohnai, Y. Goto, Y. Hayashi, M. Ueda, T. Kobayashi and M. Matsui, *Int J Hyperthermia*, 1996, **12**, 37-47.
42. E. Z. Neulander and J. Kaneti, *BJU Int*, 2011, **107**, 1685; author reply 1685-1686.
43. J. Qin, D. Priftis, R. Farina, S. L. Perry, L. Leon, J. Whitmer, K. Hoffmann, M. Tirrell and J. J. de Pablo, *ACS Macro Letters*, 2014, **3**, 565-568.
44. Y. Jho, H. Y. Yoo, Y. Lin, S. Han and D. S. Hwang, *Adv Colloid Interface Sci*, 2017, **239**, 61-73.
45. B. Yang, S. Jin, Y. Park, Y. M. Jung and H. J. Cha, *Small*, 2018, **14**, e1803377.
46. A. W. Du and M. H. Stenzel, *Biomacromolecules*, 2014, **15**, 1097-1114.
47. S. Savale, 2016, DOI: 10.20959/wjpps20164-6425, 724-742.

48. Z. Siprashvili, J. A. Reuter and P. A. Khavari, *Mol Ther*, 2004, **9**, 721-728.
49. V. P. Torchilin and A. N. Lukyanov, *Drug Discovery Today*, 2003, **8**, 259-266.
50. Y. Zhang, Y. Xiong and W. G. Yarbrough, *Cell*, 1998, **92**, 725-734.
51. A. J. Saporita, L. B. Maggi, Jr., A. J. Apicelli and J. D. Weber, *Curr Med Chem*, 2007, **14**, 1815-1827.
52. A. C. L. Mortensen, D. Spiegelberg, C. J. Brown, D. P. Lane and M. Nestor, *Front Oncol*, 2019, **9**, 923.
53. S. Nag, J. Qin, K. S. Srivenugopal, M. Wang and R. Zhang, *J Biomed Res*, 2013, **27**, 254-271.
54. D. Spiegelberg, A. C. Mortensen, S. Lundsten, C. J. Brown, D. P. Lane and M. Nestor, *Cancer Res*, 2018, **78**, 5084-5093.
55. A. Pickar-Oliver and C. A. Gersbach, *Nat Rev Mol Cell Biol*, 2019, **20**, 490-507.
56. T. Gaj, C. A. Gersbach and C. F. Barbas, 3rd, *Trends Biotechnol*, 2013, **31**, 397-405.
57. P. Mali, L. Yang, K. M. Esvelt, J. Aach, M. Guell, J. E. DiCarlo, J. E. Norville and G. M. Church, *Science*, 2013, **339**, 823-826.
58. P. D. Hsu, D. A. Scott, J. A. Weinstein, F. A. Ran, S. Konermann, V. Agarwala, Y. Li, E. J. Fine, X. Wu, O. Shalem, T. J. Cradick, L. A. Marraffini, G. Bao and F. Zhang, *Nat Biotechnol*, 2013, **31**, 827-832.
59. X. L. Li, G. H. Li, J. Fu, Y. W. Fu, L. Zhang, W. Chen, C. Arakaki, J. P. Zhang, W. Wen, M. Zhao, W. V. Chen, G. D. Botimer, D. Baylink, L. Aranda, H. Choi, R. Bechar, P. Talbot, C. K. Sun, T. Cheng and X. B. Zhang, *Nucleic Acids Res*, 2018, **46**, 10195-10215.
60. W. B. Liechty, R. L. Scheuerle, J. E. Vela Ramirez and N. A. Peppas, *Bioeng Transl Med*, 2019, **4**, 17-29.
61. F. Madani, S. Lindberg, U. Langel, S. Futaki and A. Graslund, *J Biophys*, 2011, **2011**, 414729.
62. A. Aires, S. M. Ocampo, D. Cabrera, L. d. I. Cueva, G. Salas, F. J. Teran and A. L. Cortajarena, *Journal of Materials Chemistry B*, 2015, **3**, 6239-6247.
63. J. Mariam, S. Sivakami and P. M. Dongre, *Drug Deliv*, 2016, **23**, 2668-2676.

64. S. H. Jung, S. K. Kim, S. H. Jung, E. H. Kim, S. H. Cho, K. S. Jeong, H. Seong and B. C. Shin, *Colloids Surf B Biointerfaces*, 2010, **76**, 434-440.
65. H. Nosrati, N. Sefidi, A. Sharafi, H. Danafar and H. Kheiri Manjili.
66. J. S. Suk, Q. Xu, N. Kim, J. Hanes and L. M. Ensign, *Adv Drug Deliv Rev*, 2016, **99**, 28-51.
67. J. V. Jokerst, T. Lobovkina, R. N. Zare and S. S. Gambhir, *Nanomedicine (Lond)*, 2011, **6**, 715-728.
68. A. Al-Awar, K. Kupai, M. Veszeka, G. Szucs, Z. Attieh, Z. Murlasits, S. Torok, A. Posa and C. Varga, *J Diabetes Res*, 2016, **2016**, 9051426.
69. A. J. King, *Br J Pharmacol*, 2012, **166**, 877-894.
70. C. W. Grant, S. K. Duclos, C. M. Moran-Paul, B. Yahalom, R. S. Tirabassi, G. Arreaza-Rubin, L. M. Spain and D. L. Guberski, *Comp Med*, 2012, **62**, 381-390.
71. P. Pozzilli, A. J. Signore A Fau - Williams, P. E. Williams Aj Fau - Beales and P. E. Beales.
72. L. Bakiri and E. F. Wagner, *Mol Oncol*, 2013, **7**, 206-223.
73. W. Rajewsky Mf Fau - Dauber, H. Dauber W Fau - Frankenberg and H. Frankenberg.
74. E. J. Park, J. H. Lee, G. Y. Yu, G. He, S. R. Ali, R. G. Holzer, C. H. Osterreicher, H. Takahashi and M. Karin, *Cell*, 2010, **140**, 197-208.
75. Y. Li, Z. Y. Tang and J. X. Hou, *Nat Rev Gastroenterol Hepatol*, 2011, **9**, 32-43.
76. J. Jung, *Toxicol Res*, 2014, **30**, 1-5.
77. J. Jung, S. J. Park, H. K. Chung, H. W. Kang, S. W. Lee, M. H. Seo, H. J. Park, S. Y. Song, S. Y. Jeong and E. K. Choi, *Int J Radiat Oncol Biol Phys*, 2012, **84**, e77-83.
78. G. van Meer, D. R. Voelker and G. W. Feigenson, *Nat Rev Mol Cell Biol*, 2008, **9**, 112-124.
79. E. Baykal-Caglar, E. Hassan-Zadeh, B. Saremi and J. Huang, *Biochim Biophys Acta*, 2012, **1818**, 2598-2604.
80. S. N. Jones, A. R. Hancock, H. Vogel, L. A. Donehower and A. Bradley, *Proc Natl Acad Sci U S A*, 1998, **95**, 15608-15612.

81. S. Saji, S. Nakashima S Fau - Hayashi, M. Hayashi S Fau - Toi, S. Toi M Fau - Saji, Y. Saji S Fau - Nozawa and Y. Nozawa.
82. J. D. Oliner, K. W. Kinzler, P. S. Meltzer, D. L. George and B. Vogelstein, *Nature*, 1992, **358**, 80-83.
83. C. W. Miller, A. Aslo, A. Won, M. Tan, B. Lampkin and H. P. Koeffler, *J Cancer Res Clin Oncol*, 1996, **122**, 559-565.
84. C. E. Bueso-Ramos, E. Yang Y Fau - deLeon, P. deLeon E Fau - McCown, S. A. McCown P Fau - Stass, M. Stass Sa Fau - Albitar and M. Albitar.
85. G. Teoh, M. Urashima, A. Ogata, D. Chauhan, J. A. DeCaprio, S. P. Treon, R. L. Schlossman and K. C. Anderson, *Blood*, 1997, **90**, 1982-1992.
86. Synthego, How to Select the Best CRISPR Transfection Protocol, <https://www.synthego.com/guide/how-to-use-crispr/transfection-protocols>, (accessed 28-08-2019, 2019).
87. H. Potter, *Curr Protoc Mol Biol*, 2003, **Chapter 9**, Unit 9 3.